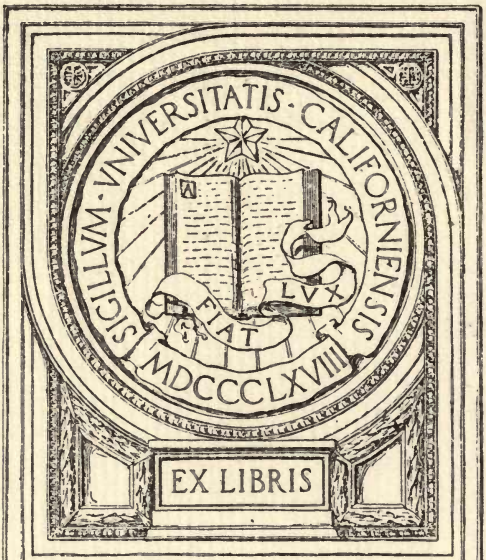

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THE BACTERIOPHAGE

ITS RÔLE IN IMMUNITY

ENGLISH EDITION

THE BACTERIOPHAGE

Its Rôle In Immunity

WITH FOURTEEN TEXT ILLUSTRATIONS

BY

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AUTHORIZED TRANSLATION

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PREFACE

In this monograph I have collected and coördinated the several notes and communications published, not only by myself but by others also, since 1917.

The study of the phenomenon was undertaken without any preconceived ideas regarding the nature of the causal principle involved. Indeed, what could it matter whether the active force was a diastase, a living germ, or some new property of the bacterium? Whatever it might be, the interest would remain the same. It was only after two years spent in investigation, with the completion of some hundreds of experiments, each one more conclusive than the preceding, that I became convinced that the cause of serial transmissible bacteriolysis could be nothing other than a living organism. And it was not until this time that my first communication on the subject was presented. Some three years later the attention of others became directed to the subject and they, for the most part, in turn suggested hypotheses as to the nature of the phenomenon differing among themselves and differing fundamentally from that which I had announced and which, in view of all of the facts and phenomena involved, is the only one which is tenable. None of these investigators have considered all of the factors and facts involved in the reaction; instead, each has selected a particular group of facts, sufficient to support his thesis, and has neglected all other experimental data such as would render his hypothesis inadmissible. It may be added that all of these hypotheses were considered prior to my first publication, and the solution of the question required many experiments indeed. In spite of the fact that the results of these experiments have been published in various memoranda none of those who have opposed my theory have refuted them, or even alluded to them. Naturally, in this monograph, these experiments will be presented, experiments which by themselves refute the interpretations of bacteriophagous activity as a diastatic action, whether the agent be derived from the organism

which is defending itself or from the bacterium which is causing the infection, both of which sources have been suggested by different authors.

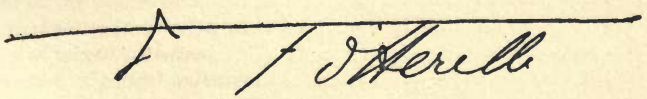
It may be that the reader will find sometimes in the course of this discussion that I have multiplied evidence by repetition, or that it is necessary to make an effort to follow certain of the experiments, so that he is lost in a maze, not only of new phenommena for which he is as yet unprepared, but also in phenomena of extreme complexity. The difficulties of exposition of the subject will readily be comprehended if we realize that up to the present time Bacteriology has been considered as a "problem of two bodies," bacterium and medium, whether the medium be the organism parasitized or a culture fluid. And this problem of the two bodies has been indeed complex. But it is of necessity much less complicated than the "problem of three bodies" with which we must now be concerned, where we must recognize the interactions between the medium—culture medium or organism parasitized,—the bacterium parasitizing this medium, and the ultramicrobial bacteriophage parasitizing the bacterium.

Paris, July 1, 1921.

PREFACE TO THE ENGLISH EDITION

The present English edition of the monograph which presents the results of my investigations on the Bacteriophage is not a simple translation of the French edition, which, appearing in October, 1921, was one of the series of monographs of the Pasteur Institute. The bibliography has been extended, and in many of the chapters new experimental evidence has been introduced, embodying work completed since the publication of the French edition. In addition, a wholly new chapter has been prepared dealing with the "Nature of the Bacteriophage," which presents a statement of and an analysis of the diverse hypotheses which have been advanced in explanation of the intimate nature of the principle. Indeed, and this is of some significance, this is the only point—purely theoretical moreover—upon which discussion of the subject turns. As for the experimental facts themselves, they have never been questioned, for all investigators who have worked with the bacteriophage have confirmed the experimental results almost in their entirety.

It is my hope that this work will be of interest to the biologist. I hope especially that it will stimulate American bacteriologists in greater and greater numbers to become interested in investigative work upon the subject; one which is new and which promises to be fruitful in theoretical and practical results. And this is my wish the more since, although working for many years in France, the signatory of these lines in the quality of a Canadian, feels himself a part of the great American family.



March 15, 1922

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PLATE 1

Multiplication of the Ultramicrobial Bacteriophage

A bacterial suspension is inoculated with one-billionth of a cubic centimeter of a filtrate containing the Bacteriophage. From time to time one-fiftieth of a cubic centimeter is taken from this suspension and spread on agar. After a period of incubation of the agar tubes the following results are obtained (reading from left to right).

Tube 1. Planting made immediately after the inoculation of the bacteriophage. A normal bacterial culture results.

Tube 2. Planting made 1¼ hours after the inoculation. A normal bacterial culture results.

Tube 3. Planting made 2½ hours after the inoculation. The layer of bacterial growth shows three colonies of the bacteriophage.

Tube 4. Planting made 3¾ hours after the inoculation. Confluent colonies of the bacteriophage are disseminated throughout the bacterial growth.

Tube 5. Planting made after 5 hours. There is no evidence of bacterial growth, the number of ultramicrobes being such that all of the bacterial elements were destroyed.

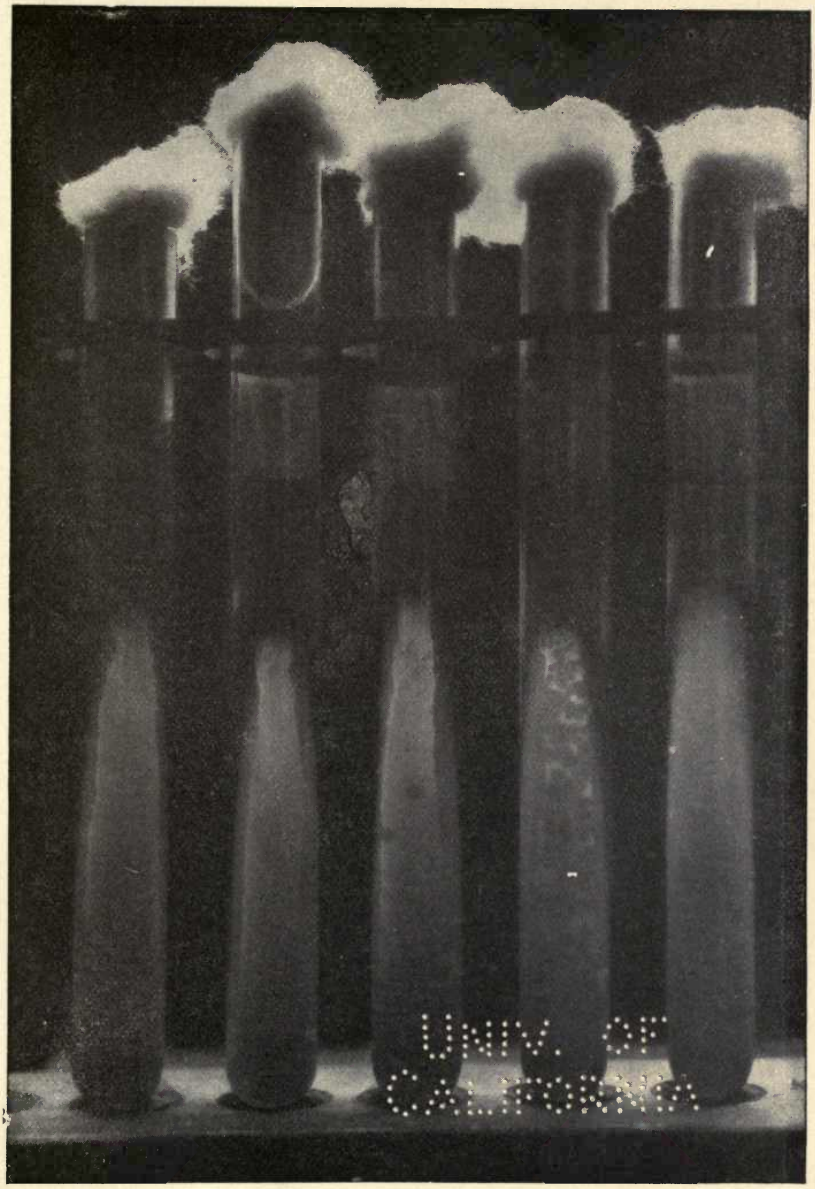

PLATE 1

PART I

THE BACTERIOPHAGE

INTRODUCTION

Historical

Examination of the scientific literature discloses but two communications bearing on the subject of the bacteriophage.

In point of priority the first is that of Hankin.[1] This author states that he detected in the waters of certain rivers of India a very marked antiseptic action, directed against bacteria in general, but against the cholera vibrio more particularly. Thus, for instance, the water of the Jumna as it leaves the town of Agra contains more than 100,000 bacteria per cubic centimeter, while at a distance of 5 kilometers further down the bacterial content is but 90 to 100.

With reference to *Vibrio cholerae* in particular, his laboratory experiments gave results as follows. Specimen "A" represents the filtered water (filtered through porcelain); specimen "B" is the same filtered water after boiling; both specimens being inoculated with a culture of *V. cholerae*.

	NUMBER OF ORGANISMS AFTER						
	0	1 hour	2 hours	3 hours	4 hours	25 hours	49 hours
Specimen A.....	2,500	1,500	1,000	500	0	0	0
Specimen B.....	5,000	4,000	6,000	10,000	6,000	10,000	36,000

The germicidal action of the water of these streams could always be detected but was present to varying degrees. It is to this antiseptic action that Hankin attributes the fact that the ingestion of the water can not be incriminated as the origin of cholera. Moreover, these streams have never been the vectors of epidemics since the propagation of such outbreaks is always from downstream upward.

[1] *L'action bactéricide des eaux de la Jumna et du Gange sur le vibrion du choléra.* Ann. de l'Inst. Pasteur, 1896, **10**, 511.

Hankin states that the antiseptic principle is destroyed by boiling and he considers himself warranted in affirming that it is a volatile substance. To my mind there is no doubt that this antiseptic action ought in reality to be assigned to the bacteriophage.

The second publication is that of Twort[2] entitled "An Investigation on the Nature of the Ultramicroscopic Viruses." In the course of his experiments upon the filtrable virus of vaccinia this author obtained on certain of his agar slants inoculated with the glycerinated vaccinal pulp, a culture of a micrococcus of which certain colonies presented a vitreous and transparent aspect. The micrococcus had been replaced by fine granules. At other times he obtained a film of growth showing spots composed of the same vitreous material. These colonies slowly spread over the entire culture, the micrococcus everywhere being transformed into granules. When a pure culture of the micrococcus was touched with a platinum wire which had previously been in contact with the vitreous material, a spot of the same nature developed and extended gradually over the whole surface. The action was feeble on cultures previously killed. The vitreous substance, when diluted, passed through a porcelain filter, for a drop of the filtrate transformed a normal healthy culture into one of the vitreous appearance. The transformation process began in isolated points and rapidly extended over the surface. However, if some portion of the normal culture never came in contact with the filtrate, the healthy growth regained the advantage and extended over the vitreous stratum but without effecting its destruction. The material of transparent and vitreous nature maintained its activity for at least six months. It resisted a temperature of 52°C. but was destroyed at 60°C.

Twort obtained similar results with an organism of the colon group, isolated from the intestinal mucosa of a dog affected with *Hundeseuche*, and with a large bacillus not belonging to the colon group isolated from the intestinal contents of an infant suffering from diarrhea. In both cases the material transformed the normal culture into matter of a vitreous transparent aspect.

[2] *An Investigation on the Nature of the Ultramicroscopic Viruses.* Lancet, 1915, ii, 1241 (December 4).

This author reviews the different hypotheses which he considered as possible causes in effecting the transformation of the cultures. The substance of vitreous appearance, he says, certainly contains an enzyme which is destroyed at 60°C. On the other hand, the material is susceptible of continued cultivation, since it can be indefinitely transplanted on a culture of the micrococcus. May it be a cultivable enzyme? May it be living protoplasm of indeterminate form? May it be an ancestral form of the micrococcus which can not be cultivated in this form but which incites the normal micrococcus to take this form of regression? Or, may it be an enzyme secreted by the micrococcus itself which produces thus its own destruction, with the formation of a new quantity of destructive enzyme? May the substance of vitreous appearance be composed of a filtrable virus which may be itself the virus of vaccinia or a filtrable virus entirely without pathogenicity derived from the air and which enters the micrococcus cultures by passing through the cotton which closes the tubes? Twort did not choose from these several hypotheses which he formulated, although it seemed to him most probable that the vitreous substance was produced by the organism, the micrococcus, itself. He indicated further, that he had no idea as to the relation which might exist between the bacillus or the micrococcus, the vitreous material, and the disease.

The phenomenon observed by Twort has been thus emphasized because it involves a serial activity, and because, on the other hand, it is hardly probable that the cause is a bacteriophage. Indeed, it has been proved that with a true bacteriophage active against *Staphylococcus albus* the phenomenon described by Twort can not be reproduced. The very peculiar characters, and indeed, the characteristics presented by the phenomenon observed by Twort render confusion of this principle with the bacteriophage impossible. The difference in thermal death points, amounting to 15°C., between the two principles (the bacteriophage becomes inactive only at about 75°C.) is alone sufficient to differentiate them. According to some experiments which I have performed the facts observed by Twort may be ascribed to a fragmentation of the bacteria; it is only necessary to use the ultramicroscope to see that the "vitreous substance" is composed of very minute cocci.

However that may be, the intensity of the bacteriophagous action is sometimes of such violence that it must have been observed by many bacteriologists in the course of their investigations even though the nature of the phenomenon and its mechanism were not understood. For example, I have been informed that in Haffkine's laboratory, it has been noted several times that cultures of the plague bacillus in bouillon underwent clarification, the medium becoming perfectly limpid within the space of a few hours. Not knowing the reason for this curious phenomenon the cultures were termed "suicides." For such reactions the bacteriophage was certainly the cause.

Another observation of the same nature is reported by Eliava, who, being in charge of the examination of the water of the Koura river at Tiflis, noted the following phenomenon. The suspected water under examination was added to a peptone solution. After a few hours of incubation a specimen taken from the surface of the medium for microscopic examination showed very numerous vibrios of normal morphology. Planted upon agar, this specimen yielded upon incubation a growth of dull appearance which microscopically appeared to be a culture of vibrios. Twelve hours later, in so far as the peptone water was concerned, all trace of the vibrios had disappeared. This experiment, repeatedly performed, always gave the same result; it was impossible to secure a culture of the vibrio. Although starting a normal development, later, within a few hours, the vibrio had disappeared. This phenomenon remained unexplained until the findings with reference to the bacteriophage were published.

In fact, it is certain that a large number of bacteriologists, indeed, it may be said all bacteriologists,—and reasons for this statement will appear in the course of this discussion,—have accidentally encountered this strange phenomenon. Seen at times in a fluid medium, at other times on a solid medium, such reactions have repeatedly been observed but their study has been neglected since their importance was not recognized.

FUNDAMENTAL EXPERIMENT

The experiment which served as a point of departure for subsequent work was as follows. An adult, suffering with a severe

dysentery (*B. dysenteriae Shiga*) was under treatment in the Pasteur Hospital. Each day an examination of the feces from this patient was made by the inoculation of bouillon with some of the fecal material. After incubation at 37°C. for over night the growth was filtered through a Chamberland filter. To a second tube of broth, previously inoculated with a culture of the Shiga bacillus, twelve drops of the filtrate were added and the culture so treated was returned to the incubator. Throughout the period of the infection, all of the tubes, prepared each day in the same manner, yielded normal growths of the dysentery bacillus. One day, examination of the tube prepared the day before showed no growth, and investigation showed that the patient presented symptoms indicative of marked improvement. Definite convalescence rapidly followed. The bouillon which had been inoculated with both culture and filtrate was to all appearances sterile, and to this was again added a suspension of Shiga bacilli taken from a young agar culture. The inoculation was sufficiently heavy to present a definite turbidity, but after incubation for twelve hours it was again clear. The excreta from which the filtrates were prepared contained, then, a principle which dissolved the dysentery organisms.

When a drop of the lysed culture was added to a young bouillon culture of the Shiga bacilli this culture in turn became dissolved. In the same way, several successive passages were accomplished, introducing each time a drop of the culture previously lysed into a fresh culture of the Shiga strain. Instead of losing in potency through such passages it increased in lytic capacity; the dissolving action being accomplished more and more rapidly. From this it was evident that the lytic principle derived from the excreta was capable of cultivation in series.

When a very minute amount (0.00,001 cc.) of one of these lysed cultures was added to a young broth culture of the Shiga bacillus and then this mixture was tested immediately and again after incubation periods of one, two, and three hours, a drop of the material being inoculated on to agar slants, the latter showed after incubation very interesting characteristics. In the first tube thus inoculated the agar was covered by a normal film of dysentery bacilli, but with two circular areas about 2 mm. in

diameter entirely free of any evidence of bacterial growth. The second tube, inoculated with material taken one hour after the admixture of culture and lytic agent, presented six of the clear plaques. In the third tube there were about 100; and finally, on the fourth there was no apparent growth.

Here was new evidence that the lytic principle actually multiplied, and furthermore, that this principle actually existed in particulate form. The element from which the lytic phenomenon originated was composed of masses which were deposited upon the agar in definite points. Each mass was capable of multiplication since, independent of the action in series, it yielded a colony. It could be considered as nothing other than a ferment or a living being parasitic on the bacteria. But it is impossible to comprehend a soluble ferment—a diastase—as multiplying in the form of granulations and concentrating its activities in limited, clearly defined points.

As we will see in the course of this work all the experiments, without a single exception, are in accord in showing that the principle acts as a virus; and, indeed, as a virus which presents all the chief characteristics of organisms, including the fixation of complement with an antiserum.

In employing the word "microbe," or better "ultramicrobe," following the happy expression of Calmette, I give to this word its true meaning: "minute living being," without any suggestion as to what kingdom it may belong. Is it a bacterium, a protozoon, or a yeast? That must be ignored. Its dimensions are too small to permit the determination of this question by direct microscopic observation. May it be a cell, infinitely small, an *organite*, derived from a superior organism; a cell indefinitely cultivable in series *in vitro* at the expense of bacteria and maintaining itself as an autonomous being? This is hardly probable, but it is never permissible to reject *a priori* any conception which accords with the known facts. Experiment has shown that this lytic principle, which has been termed *Bacteriophagum intestinale* or bacteriophage, is a particle which proliferates at the expense of bacteria; and, as a result, is capable of assimilation and is indefinitely cultivable in series *in vitro* in the form of a filtrable substance. It behaves like living matter because assimilation and reproduction are fundamental characteristics of life.

This introductory discussion should not be concluded without a word on the subject of the term "Bacteriophage," a term which has been criticized. The suffix "phage" is not used in its strict sense of "to eat," but in that of "developing at the expense of;" a sense that is frequently used elsewhere in scientific terminology. Certain protozoa, for example, are parasitized by the *Nucleophaga* which develop within the interior of the nucleus. This is precisely the interpretation to be given the term "phage" in the word "Bacteriophage."

CHAPTER I

Bacteriolysis

Bacteriolysis. Technic for Isolating the Bacteriophage. Enhancement of Virulence. Technic for Enumeration. Multiplication at the Expense of the Bacteria in a Fluid Medium. The Bacteriophage; an Obligatory Parasite. Effect of the Condition of the Bacterium. Effect of the Medium. Cultivation on Solid Media; Isolated Colonies. Effect of the Concentration of Bacteria in the Medium. Destructive Action of the Secretory Products of the Bacteriophage. Effect of External Physical Conditions. Effect of Antiseptics. The Soluble Bacterial Substance. The Bacteriophagous Ultramicrobe; an Internal Parasite. Bacteriolysis under the Microscope.

BACTERIOLYSIS

It is expedient to define, at the beginning of this work, what is meant by the word "bacteriolysis" for it is a scientific term used in a somewhat equivocal manner.

The term "autolysis" was introduced into science by Jacoby as a substitute for the word "autophagy" which had previously been employed to designate the process of softening; the tendency toward a liquefaction, more or less marked, such as is produced by a yeast isolated upon a nutrient medium. The term autophagy, which presumed nothing as to the final condition of the process, is more suitable certainly, than that of autolysis. Etymologically the latter signifies auto-dissolution, whereas as a matter of fact, the process of autolysis, as it occurs with bacteria and yeasts even if prolonged for several months, never results in a complete cellular dissolution. The end product is a semifluid mass, which, examined microscopically, shows cellular debris along with a greater or less number of cells more or less profoundly modified. The degree of disintegration depends somewhat upon the type of bacterial cells employed. Autolysis, then, is characterized, not by an actual dissolution, but by a disintegration, a cellular fragmentation with a partial dissolution of certain elements. Even in the most favorable cases, when the autolysis is considered

complete, it is attended by the formation of an amorphous mass insoluble in the fluid. If the significance of the term "lysis" is indeed exact in autolysis where there is a partial dissolution, it is by no means the same in "bacteriolysis" as this is understood by many authors. Treatises dealing with immunity speak of "bacteriolytic sera," realizing explicitly that the reactions between antigen and antibody never consist of a digestion. How then, under these conditions, is it possible to have a dissolution? And, in fact, none is ever observed.

The action manifested by the bacteriophage is wholly different. It comprises phenomena of which the final result is a digestion such as leads to a *total* dissolution of the bacterial bodies. It is a bacteriolysis in the true sense. At first I considered designating this new phenomenon by a new word, "bacteriophagy" for example, since the word bacteriolysis is often employed to designate processes differing entirely from dissolution. But since the use of too frequent neologisms might distract the reader I have felt that the abuse which has been made of the term "bacteriolysis" may be only a transitory one and that the passage of time will soon cause to be forgotten the phenomenon of so-called bacteriolysis without a dissolution of the bacteria.

To summarize: the phenomena which we will consider have nothing in common with that which is usually designated by the terms "lysis" and "bacteriolysis." Here, the term "lysis" should always be taken in its strict etymological sense of a complete dissolution. A bacterial culture in bouillon or a suspension of bacteria in a fluid where bacteriolysis, as we understand it, takes place, completely clears, without residue. The fluid becomes as limpid as it was prior to its inoculation with culture.

TECHNIC FOR THE ISOLATION OF THE BACTERIOPHAGE

All bacteriological laboratories possess the equipment required for the isolation and cultivation of the bacteriophage. For isolation a filtering apparatus is indispensable. (I have employed the model of Martin, with Chamberland L_2 and L_3 bougies.) For culture media a peptone bouillon, or such a bouillon incorporated into a 2 per cent agar, is adequate.

The active bacteriophage may be sought for in materials which require some preliminary treatment, since in their natural physical state they may not lend themselves readily to filtration. The following types of material may be examined as a source of the bacteriophage.

1. A sterile fluid; sterile in the sense in which the word[1] is usually employed; for example, blood, or an organic fluid collected aseptically. With such no treatment is necessary.

2. The material to be examined may be a clear liquid but not sterile. With this, filtration will eliminate the bacteria while the bacteriophage passes through into the filtrate.

3. The material may show a homogeneous turbidity; as a bacterial culture. Here, direct filtration results in an early occlusion of the pores of the bougie. Thus, it is desirable to resort to a preliminary filtration. The following method of treatment is most satisfactory.

Provide a funnel with a folded filter paper sufficiently large to receive at one time the entire volume to be filtered. Fill the filter with water to which has been added a small amount of infusorial earth. When the water has passed through, the paper is left coated with a thin layer of the infusorial earth, thus rendering the paper less permeable. Through this the material to be examined is filtered prior to filtration through the bougie.

4. The material may be a fluid holding in suspension organic particles, or it may be matter more or less solid in nature. This is the type of substance most frequently examined; such as fecal

[1] In the course of this work I find myself frequently in difficulty in the exposition of facts because of certain expressions which have been appropriated to describe certain conditions. I shall apply the word *sterile* to a medium which contains no visible microscopic organisms or organisms capable of cultivation upon artificial media. An *ultrasterile* medium is one which contains no ultramicrobes. A substance containing the virus of measles, for example, is *sterile* but not *ultrasterile*, since it is still capable of transmitting measles although it contains nothing visible or cultivable. Media containing the bacteriophage are likewise *sterile* in the bacteriological sense of the word, since they are perfectly limpid and since the germ which they contain can not be cultivated alone upon artificial media of any kind. But such a medium is not *ultrasterile*, for it does contain a principle which will grow at the expense of bacteria, just as the virus of measles will grow at the expense of higher organisms.

material more or less fluid, pasty, or solid; or excreta admixed to a greater or less degree with earth, organic debris, etc. In such a case it is necessary to disintegrate as completely as possible the material to be examined.

To effect such a disintegration the most simple procedure consists in carefully suspending the material in bouillon, about 5 gm. to 50 cc. of the medium, and incubating this suspension at 37°C. for from twelve to eighteen hours. The bacterial fermentations which ensue, because of the diverse organisms introduced into the medium, lead to a sufficient disintegration. Upon removal from the incubator the material may be treated, as indicated above, by filtration through infusorial earth and a bougie.

If the material under examination contains the bacteriophage and has been subjected to filtration, the ultramicrobe will be found in the filtrate. The methods of purification outlined above are applicable to two purposes, (A) the detection of a bacteriophage active toward a given bacterial type, and (B) to test the activity of a bacteriophage, either upon diverse organisms or against a single bacterial strain of indeterminate type.

A. The first case is the more simple and will be considered first, taking as an example the detection of a bacteriophage active against *B. dysenteriae Shiga*. The day before the test is to be made an agar slant is inoculated with the dysentery strain. From this fresh culture, on the day of the test, four tubes of peptone broth are inoculated.[2] To the first of these tubes is added one drop of the filtrate, to the second, ten drops, and to the third, two cubic centimeters. One tube, simply inoculated with the dysentery organism, serves as a control. The tubes are incubated at 37°C. After twelve to eighteen hours one of several results may be observed: the three tubes (in addition to the control tube) may all show a turbidity due to the growth of the dysentery organism, only one or two of the tubes may be turbid, or the three tubes may be clear.

[2] As will be shown later this bouillon should be alkaline in reaction. The ordinary neutral bouillon (I have always used by preference the bouillon of Martin) to which is added 6 cc. of N/1 NaOH per liter is perfectly satisfactory. This is, moreover, the degree of alkalinity most frequently employed in bacteriological work.

1. All three tubes are turbid. From such a result it may not be concluded that an active bacteriophage is not present, for if a complete lysis of the bacteria is taken as the only criterion for determining its presence the bacteriophage will, in a majority of cases, be overlooked. Lysis is but a single fact in the midst of a very complex group of phenomena. If the three tubes are turbid take about 0.02 cc. from each of the tubes by means of a platinum loop and spread over the surfaces of three tubes of slanted agar. If, after incubation, these tubes present normal cultures of the dysentery bacillus the result of the test is negative. That is, the original material did not contain an active bacteriophage for *B. dysenteriae Shiga*. The presence of the bacteriophage in active form is indicated by an abnormal appearance of the growth as it develops on the agar. In accordance with the number of bacteriophagous ultramicrobes present the aspect of the culture will vary. The layer of bacillary growth may show one, or several, circular areas where the surface of the agar appears devoid of growth. Or, the culture may appear broken up, or corroded, as a result of the confluence of the areas. Indeed, there may be only fragments of culture or even isolated colonies remaining. When the number of ultramicrobes is still greater the slant may be free of any evidence of bacterial growth.

As will be shown, each strain of bacteriophage is endowed with an individual degree of virulence, the word "virulence" to be taken in its true meaning, that is, "ability to multiply at the expense of the parasitized being." Certain races of the bacteriophage multiply rapidly, others increase but slowly. The first possess a high degree of virulence toward the bacterium provided for their development; the second possess but a feeble virulence. We will elsewhere return to this subject of the virulence of the bacteriophage. It is mentioned here simply to explain the reason why strains of the bacteriophage show variability in growth when isolation is attempted. The diameter of the clear areas, varying with the individual strain of bacteriophage from a fraction of a millimeter (the bacterial growth appears as though sprinkled with pin point areas) up to 4 to 5 mm., gives a measure of virulence; the larger the area the higher the virulence. We shall see that whatever the virulence of a particular

strain of the bacteriophage when it comes from the organism it can be enhanced *in vitro*.

2. The first or first and second tubes only give a culture of the dysentery bacillus. Here the filtrate contains a bacteriophage of average or high activity. It is only necessary to proceed as is indicated in the following case to secure areas of bacteriophagous growth on agar.

3. All three tubes remain clear. This indicates the presence of a bacteriophage of extremely high activity. Confirmation is simple. It consists in taking three tubes of bouillon, in adding to them a suspension of young bacilli taken from an agar slant in a concentration to give a slight turbidity. Introduce into each of these tubes a drop of the fluid from each of the three tubes which had remained clear, shake, and then immediately distribute a loopful of each upon the surface of an agar slant. Both sets of tubes are incubated. After twelve to eighteen hours the three bouillon tubes will be limpid; the three agar slants will present the appearance already described for cultures of *B. dysenteriae* admixed with the bacteriophage.

B. dysenteriae Shiga has been taken as an example, although whatever may be the bacterial type against which an active bacteriophage is sought the technic for isolation remains essentially the same. The medium is inoculated with the bacterium in question, as, for example, with *B. pestis* if a bacteriophage active for the plague bacillus is sought.

B. Instead of determining if a given material contains a bacteriophage active for a certain bacterium, it may be desirable to ascertain if a bacteriophage which has been isolated possesses an activity for several bacterial types at the same time. In this instance three tubes with each of the bacterial types to be investigated may be prepared. Thus, to investigate the activity of an intestinal bacteriophage against *B. dysenteriae Shiga*, *B. dysenteriae Flexner*, *B. dysenteriae Hiss*, *B. typhosus*, and *B. coli*, five series of three tubes are prepared. The first set is inoculated with *B. dysenteriae Shiga*, the second with *B. dysenteriae Flexner*, the third with the *Hiss* strain, and the fourth and fifth sets with *B. typhosus* and *B. coli* respectively. To the first tube of each series one drop of the filtrate is added, to the second, ten

drops, and to the third, two cubic centimeters. With each set the procedure is that already indicated for the Shiga organism.

In routine work a single tube can be used in place of the three tubes, and to this ten drops of the filtrate is added, but with this simplified technic the danger lies in the fact that a bacteriophage of weak activity may not be detected.[3]

TECHNIC FOR ENHANCING VIRULENCE

It has already been stated that a bacteriophage may be present even though it is unable to induce the slightest macroscopic evidence of the lysis of a bacterial suspension. Indeed, this is the situation most frequently encountered in the process of isolation However, it is, as a rule, easy to increase the activity of such a bacteriophage. One of the following methods suffices:

[3] Mention may be made here of a method of Bordet and Ciuca, and it is upon this procedure, moreover, that these authors have based their theory of hereditary lysis. They inoculate a guinea pig intraperitoneally at three or four different times at intervals of a few days with a culture of *B. coli*. The day after the last injection, according to them, it is only necessary to wash out the peritoneal cavity, whereupon the principle giving rise to "hereditary lysis" is found in the exudate. From their first communication on the subject it is evident that they consider this observation a specific example of a general law—which indeed would be one of the *sine qua non* conditions for the validity of their theory—that an injection of any bacterium causes the organism to respond with the production of a principle which gives birth to the phenomenon of lysis in series. They stated that they would shortly announce the results secured with diverse bacteria but this communication has never appeared. I have tried without success, as have several other investigators, to duplicate the results described by Bordet and Ciuca. In reality, in this experiment, there has been a passage of the anticoli bacteriophage, which, as experiment shows, is normally present in the guinea pig intestine, into the peritoneal cavity as a result of the irritation induced by the inoculations. After its appearance in the peritoneum it multiplies there by virtue of the *B. coli* inoculated. The correctness of this interpretation is vouched for by the fact that the results reported by Bordet and Ciuca can be secured if, a few hours before the intraperitoneal injection of bacterial culture, the bacteriophage active for this bacterial type is given the animal *per os*. The active bacteriophage found in the intestine passes through into the peritoneal cavity. The method of Bordet and Ciuca gives results only by accident, only when an active bacteriophage was previously present in the intestine. Thus, all of the conclusions of these authors fall *ipso facto*. We will return elsewhere to this question (Chapter VI, Nature of the Bacteriophage).

When the agar inoculation has shown that a bouillon suspension contains an active bacteriophage this suspension is filtered through infusorial earth and then through a bougie. A slightly turbid suspension is prepared, using the bacterial strain against which the bacteriophage has shown some activity, and into this suspension are introduced some four or five drops of the filtrate. After an incubation period of twenty-four hours at 37°C., if lysis has not been produced, this second bacterial suspension is filtered as before and a third suspension is inoculated with four or five drops of the filtrate. Such transfers are continued until evident lysis occurs. During the process it is easy to verify the presence of the bacteriophage in each passage, and to detect any increase in virulence, simply by spreading the successive cultures on agar slants. Comparison of the cultures secured with each passage reflects the degree of virulence. For example, the agar growth obtained from the first passage shows a culture growth with ten plaques, the second passage shows 100, with the third the layer of bacillary growth is broken up with an abundance of the areas, while with the fourth passage only a few isolated colonies of bacteria are seen. It can be readily seen that the virulence of the bacteriophage, that is, its ability to develop at the expense of the bacteria, increases with each transfer until a point is reached where lysis of the suspension is obtained.

Successive transfers can be made upon agar slants, taking the material from a tube showing the clear areas. With a platinum wire material can be removed from the bacterial growth bordering on a plaque and inoculated on a sterile slant. A second, third, and fourth (or as many as may be desired) transfer from agar to agar can be made. When a condition is reached where the agar growth shows only fragments of bacterial culture the surface of this tube is carefully washed off and filtered through infusorial earth and a bougie. In the filtrate is found a bacteriophage sufficiently active to produce lysis of a bouillon suspension.

As we shall see, the bacteriophage is not destroyed at 65°C., that is, at a temperature above the thermal death point of most non-sporulating bacteria. Thus, instead of filtration the application of heat may be employed. Heating at 58 to 60°C. for thirty minutes will kill the bacteria and not harm the bacterio-

phage. However, filtration has always appeared to give more satisfactory results. In a later chapter, under a paragraph entitled "Multiple Cultures" a third procedure will be considered.

In certain cases the virulence of the bacteriophage can be increased *in vivo*. A guinea pig is injected intraperitoneally with two cubic centimeters of the filtrate containing the bacteriophage whose virulence it is desired to increase and with a few cubic centimeters of the bacterial culture against which the bacteriophage is active. After twelve to eighteen hours, ten cubic centimeters of sterile bouillon is injected into the peritoneum and a few minutes later the peritoneal exudate is removed by puncture with a trocar. The fluid is collected in a few cubic centimeters of citrate solution and after a few hours' incubation the material is filtered (infusorial earth and bougie). This filtrate frequently shows that a bacteriophage is present which is significantly more virulent than that which was introduced into the guinea pig.

Usually it is relatively easy to increase the virulence of a weak strain of the bacteriophage, but at times it may become very difficult, particularly when working with strains active against the Gram-positive cocci. In such cases it is necessary to effect a great number of passages, and there is considerable risk of losing the bacteriophage altogether, particularly during the first few passages. I might cite as an example an anti-staphylococcic strain with which Eliava was forced to make passages during four months in order to obtain sufficient virulence to induce complete lysis of a suspension containing 500 million staphylococci per cubic centimeter.

ENUMERATION OF THE BACTERIOPHAGOUS ULTRAMICROBES

A trace of a filtrate containing a bacteriophage very active for a given bacterium introduced into a broth suspension of this bacterium causes a lysis of the organisms there present within a few hours. The medium becomes as clear as broth which has never been inoculated. A trace of the lysed suspension introduced into a new suspension similar to the first causes a similar lysis, a trace of this second lysed suspension introduced into a third tube reproduces the same phenomenon, and so on. During the past three years with a single strain daily passages have

been made, sometimes two or three passages on a single day, introducing in each transfer about 0.001 cc. of the last tube lysed into a fresh suspension. After more than 1500 passages the lysed suspension of the last tube was as active, even more active, than the filtrate which served to start the phenomenon in the first tube of the series.

A suspension once lysed does not contain any living bacteria. On the other hand, the amount of bacteriophagous ultramicrobes introduced to start the lysis is increased, since the new lysate is as active as was the preceding one. The lysed suspension has become what can be called, literally, a culture of the bacteriophage.

It has been previously stated that the inoculation of agar with a bacterial suspension to which has been added a small amount of fluid containing the active principle gives a bacterial culture studded with clear areas. This observation suggests a means of determining with approximate exactness the phenomenon of multiplication of ultramicrobes, since each area undoubtedly indicates the point at which, during the inoculation, there was deposited an active element,—an ultramicrobe. It is only necessary to work with measured quantities to ascertain the number of active germs in a fluid.

From an abundance of experiments a single one showing the method of counting is taken. To avoid repetition it may be stated that "suspension of *B. dysenteriae Shiga*" is to be understood.

A series of tubes is prepared, once for all, by any standard procedure, containing *B. dysenteriae Shiga* suspensions of the following counts: 100, 200, 250, 300, and 400 million bacilli per cubic centimeter. These suspensions are stabilized by the addition of a small amount of formol and the tubes are sealed with the blowpipe.

The suspension to be subjected to lysis should be taken by preference from a young growth on agar. A concentrated suspension is prepared by adding one or two cubic centimeters of bouillon to the agar slant and allowing the tube to remain inclined for a few minutes in such a way that the whole bacterial growth is under the fluid. With shaking, a perfect suspension

is secured. With a pipette a certain quantity of this concentrated suspension is added, drop by drop, into a tube of bouillon until the turbidity corresponds to that of the 250 million control suspension. This approximation is adequate for routine practice, giving a suspension which contains about 250 million bacilli per cubic centimeter. This is the suspension to be used. A young broth culture can be utilized, but the other suspension, more accurately adjusted, is to be preferred.

Experiment I. To a tube containing 10 cc. of a suspension of *B. dysenteriae Shiga* is added 0.00,002 cc. of a culture of the bacteriophage ten days old, i.e., a Shiga suspension that has been lysed for ten days. The tube is shaken to ensure even distribution and with a tared platinum loop 0.01 cc. of the liquid is removed and spread as uniformly as possible, by rubbing, over the surface of an agar slant. This tube is incubated at 37°C. After eighteen hours it presents a Shiga culture studded with 51 plaques.

The calculation is simple. The 10 cc. of suspension received 0.00,002 cc. of the bacteriophage culture, or 0.00,000,2 cc. for each cubic centimeter of medium. The amount of material taken for planting on agar was 0.01 cc. This contained, therefore, 0.00,000,002 cc. of the original bacteriophage culture. Upon agar this 0.01 cc. gave 51 clear areas, or 51 colonies, each one of which developed from a single bacteriophagous germ deposited upon the surface. Fifty-one germs for 0.00,000,002 cc. represent 2,550,000,000 germs per cubic centimeter. This is, therefore, the content of the culture of the bacteriophage which was used to inoculate the bacterial suspension.

The technic for counting the ultramicroscopic bacteriophage hardly differs from that used in counting ordinary bacteria. With the latter isolated colonies are secured on a surface otherwise sterile. With the ultramicrobe, clear areas representing colonies are obtained superimposed upon a layer of bacterial growth. Counting can not be effected otherwise, since the bacteriophagous ultramicrobe is only able to develop upon the bacteria which constitute its culture medium.

Each plaque represents a colony of the bacteriophage. This is indisputable, for if the centre of such an area is touched with the point of a drawn-out capillary pipette and this pipette is dropped into a suspension of dysentery bacilli, the culture, when

planted upon agar, reveals characteristic clear plaques. If, in the bouillon, lysis is allowed to proceed for some hours, there are more plaques. The plaque, then, to all appearances sterile, is in reality a colony of the bacteriophage.

MULTIPLICATION OF THE ULTRAMICROSCOPIC BACTERIOPHAGE

The multiplication of the bacteriophage in the course of its activity can be followed by the method of counting. With a definite suspension of Shiga bacilli, always 250 million per cubic centimeter, two extreme cases are very interesting: (1) that which occurs when a large number of ultramicrobes are introduced,[4] and (2) what transpires when but few, or only a single one, is inoculated.

1. Mass inoculation. Ten cubic centimeters of a suspension of Shiga organisms are inoculated with 0.04 cc. of the culture of the bacteriophage. The culture of bacteriophage contains 3000 million germs per cubic centimeter. Observed macroscopically from time to time, it will be seen that the turbidity gradually increases up to about the third hour,[5] and from that time the liquid clears little by little. Between the fourth and sixth hours from the time of the inoculation the suspension has become limpid.

[4] As already stated, study of the bacteriophage is always the study of a complex problem, because it is essential to consider the mutual actions and reactions of three variable factors—medium, bacterium, and bacteriophage. The complexity is rendered more difficult to express clearly because of the lack of suitable words. Here, for example, what shall we term the act of introducing a definite quantity of the bacteriophage into a bacterial culture? Obviously the term "inoculation" can be employed, but the medium has already been inoculated with the bacterium; thus an equivocal or ambiguous meaning may result. The proper term would be "contamination" but unfortunately this term has already been appropriated in bacteriology as a synonym for "pollution". The term "inoculation", then, must be employed. A culture medium may be "seeded" with bacteria, and then "inoculated" with the bacteriophage.

[5] Frequently it will be observed, and the conditions for this reaction are not exactly determined, that the dissolution of the bacteria is preceded by a very marked agglutination. This reaction is noted particularly when the bacterial culture is inoculated with a relatively large amount (twelve drops for example) of a bacteriophage of average virulence.

If, immediately after the inoculation, and at regular intervals for six hours, a loopful of the liquid is planted on agar, all the tubes remain sterile. One would readily think that the absence of colonies of Shiga means that they have been killed with the first contact with the inoculated bacteriophage. But this is not the case for if instead of planting agar with the suspension as such, it is previously diluted to 1:1000 with bouillon, and then this dilution is planted on agar numerous colonies of *B. dysenteriae* develop. The bacilli have by no means been killed. If the transfer of the undiluted suspension to agar remains sterile it is simply because the agar has been planted simultaneously with many living Shiga bacilli and also with a large number of the ultramicrobes. Indeed, the bacilli have commenced to multiply in the nutritive medium but the bacteriophage has not been inactive. Finding the bacilli which they parasitize within reach, the ultramicrobes act upon them, reproduce, and inhibit the bacillary growth. In the other case, with the suspension diluted to 1:1000, the bacilli and the bacteriophagous germ, a thousand times less numerous, are separated by spaces sufficiently great so that immediate interaction is less readily accomplished. This allows the bacilli which are outside of the immediate neighborhood of an ultramicrobe to multiply and to form colonies.

A second dilution (1:1000) of the suspension, made after the latter has been incubated for an hour, more often remains sterile when transferred to agar and only rare colonies develop. After two hours of incubation, cultures on agar always remain sterile. At this time all the bacilli contained in the suspension have been attacked and none of them are able to reproduce.

2. Inoculation with few anti-microbial elements. Six tubes of the Shiga bacillus suspension are inoculated with the bacteriophagous culture (containing 3000 million per cubic centimeter) in such a way that each tube receives one six-millionth of a cubic centimeter. When incubated, four give normal cultures of *B. dysenteriae* and all subcultures on agar give normal growths. They are therefore without interest to us. The other two, however, which have each received probably one, certainly not more than two ultramicrobes, show the following picture: The suspension becomes more and more turbid. After two hours at

37°C. the opacity is about two times as great as at the beginning; after three hours, it is about two and one-half times as great; after four hours, about three times; and then it begins to diminish, so that after five hours the density is about twice as great as at the beginning of the incubation. This clearing continues gradually, so that after fourteen hours the culture is almost entirely clear. If immediately after the inoculation with the bacteriophage and then every thirty minutes, 0.02 cc. of each of these two suspensions is transferred to agar slants, these tubes will show, after incubation, the following:

Plantings after one-half, one, one and one-half, and two hours yield normal growths of *B. dysenteriae Shiga*. After two and one-half hours the subcultures show three plaques in one tube, five in the other (average four). Therefore, after two and one-half hours the inoculated suspension contains 2000 bacteriophagous ultramicrobes.

After three hours the tubes show five and four respectively. Hence, there has been no material increase between two and one-half and three hours.

The three and one-half hour plantings show nine and five areas (average seven). The number of bacteriophagous elements has slightly increased.

After four hours, the agar tubes show 101 and 111 plaques respectively (average 105). After four hours, therefore, the number of ultramicrobes is between fifty and sixty thousand.

After four and one-half hours, the counts are 145 and 160 (average 152), indicating that the suspension contains 75,000; a number but slightly different from the count after four hours.

After five hours, the agar tubes are sterile. When diluted to 1:1000 in a suspension of Shiga bacilli and transferred immediately to agar in the same way, the tubes give four and six areas. Thus, it appears that after five hours the suspension contains about 1,500,000 bacteriophagous germs.

From this it is readily apparent that the multiplication of the ultramicrobes is extremely rapid, and, what is most remarkable, it is associated with successive jumps, each augmentation being separated by an interval of about seventy-five minutes. We will refer elsewhere to this experiment when we consider the mode of reproduction of the bacteriophage.

This experiment shows that it is only necessary to have a single bacteriophage present in a bacterial suspension to produce a complete lysis of the bacteria, provided the strain of bacteriophage is of maximum activity.

Needless to say, such experiments have been repeated many times, always with results comparable to those cited. Indeed, this statement holds for all of the experiments reported in this monograph—all have been repeated.

The attention of investigators should be called to this particular point, namely, that once a strain of bacteriophage of sufficient virulence has been obtained, the end results of the experiments are always the same, without exception—an increase in the number of ultramicrobes inoculated and a complete lysis of the bacterial suspension. Repeating the same experiment several times with the same strain of bacteriophage, employing always the same conditions of medium and temperature, the proliferation of the ultramicrobe progresses in the same manner and lysis is effected in the same length of time. But if one is making a comparative study of different strains, although all are endowed with high activity, certain differences are noted. With one strain complete lysis will be obtained after three and one-half hours (this is the shortest period thus far observed), with another only after fourteen hours, all the conditions being the same. In a word, and this observation likewise applies to all of the experiments here reported, the phenomenon always proceeds as has been indicated. The time alone may vary. The ultramicroscopic bacteriophage is a living being, and as such, the processes which it carries out can not go on with the regularity of a diastatic action.

Experiment II. Here is, to cite an example, an experiment conducted with another strain of bacteriophage. It will be noted that lysis is effected much more quickly than in the instance given above. The suspension of Shiga bacilli is made in bouillon previously warmed to 38°C., and the suspension is inoculated with 0.00,01 cc. of the culture of bacteriophage. The macroscopic appearance showed that:—After two hours the suspension is three times as turbid as at first.

After two and one-half hours it is about three and one-half times as turbid.

After two and three-quarter hours it is about three times as turbid as at first.

After three hours the turbidity is hardly apparent.

In this experiment the lysis was almost entirely accomplished within a space of fifteen minutes, that is, during the period of time between two and three-quarters and three hours after the inoculation.

A single ultramicroscopic bacteriophage is, therefore, adequate to provoke lysis. If successive dilutions of a culture of the bacteriophage are prepared, one drop of the culture into a tube of sterile bouillon, one drop of this first dilution into a second tube, a drop of the second into a third, and so on, and if into each of these dilutions a fixed quantity of a concentrated Shiga culture is introduced, lysis is secured in all tubes which have received at least one ultramicrobe. This is usually the first four tubes of the series. The remaining tubes will show a normal growth of the Shiga bacillus. Since we have been able to make counts of the bacteriophage and recognize the rapidity with which even a single ultramicrobe can proliferate and bring about lysis, these observations are self-explanatory. Without this means of investigation one would be liable to commit a serious error and to conclude that the sterile bouillon of the first four tubes had contained a culture of the bacteriophage other than that introduced in preparing the dilutions. Incidentally, this error has been committed by certain authors. In reality, while there has been a dilution, the diluted culture was active just so long as there was to be found a single ultramicrobe.

Recognizing the number of ultramicrobes in a lysed suspension, which has, in effect, become a culture of the bacteriophage, and the value of the dilution, it can be mathematically determined whether a bacterial suspension inoculated with such a dilution will undergo lysis or not. This test has been performed by experiment more than a hundred times with very diverse strains of the bacteriophage.

THE BACTERIOPHAGE: AN OBLIGATORY PARASITE

Whatever may be the medium employed, in the absence of a bacterium for which the bacteriophage is active, multiplication of the ultramicrobes never takes place. And this remains true even if inoculated into a medium containing, instead of living bacteria, organisms that have been killed by any procedure what-

soever. All experiments have been uniformly negative in attempting to obtain multiplication of the bacteriophage by contact of the ultramicrobe with bacteria killed by age, by heat, by chloroform, by thymol, by the essences of cinnamon and mustard, by alcohol, by bichloride of mercury, by phenol, by sulfuric and hydrochloric acids, and by iodine. With such suspensions no action whatever is secured;—no lysis and no culture of ultramicrobes.

The bacteriophage is an obligatory parasite, multiplying only at the expense of living bacteria.

An experiment of the following nature is interesting in that it shows that the bacteriophage will attack only normal bacteria. The bacteria are suspended in a medium containing an antiseptic in a quantity so small that the bacteria will only be killed after a time exceeding that required for the lytic process of the bacteriophage. In such a case the bacteria are not affected by the bacteriophage and the latter fail entirely to proliferate. The antiseptic selected was, by intention, one without action on the diastases,—sodium fluoride.

In a one per cent solution of sodium fluoride in bouillon the Shiga bacilli are still cultivable after thirty-six hours, a time more than adequate for the bacteriophage to manifest its lytic activity and to multiply. Furthermore, if transfers in series are made with such a suspension, inoculating the first tube with a drop of the bacteriophage culture; the second, after incubation for twenty-four hours, with a drop of the first; the third with a drop of the second, and so on, it will be found that the bacteriophage disappears with the transfer from the second to the third tubes of the series (this can be confirmed by placing a drop of each tube into a suspension of normal bacilli). Controlling this procedure with a second series, using pure bouillon or even sterile water, it will be found that here likewise the bacteriophage will not be present in the third or fourth tube. This is, as will be seen later, simply a result of dilution. The bacteriophagous germ, therefore, can not be cultivated in a suspension containing fluoride, in sterile bouillon, or in pure water.

Moreover, it has been shown that it is solely because of the medium into which it is introduced that the bacillus is not sub-

ject to attack. For after a twenty-four hour stay in fluoride bouillon a normal culture will be secured by transfer from this medium into ordinary bouillon, and this culture is normally lysed by the bacteriophage.

This will be discussed later, accepting for the moment that here is a medium in which the Shiga organism remains alive for at least thirty-six hours, in which the bacteriophage likewise remains alive, which exerts no inhibitory action on the diastases, but in which the bacteriophage fails to multiply.

THE EFFECT OF THE CONDITION OF THE BACTERIA

These experiments have been conducted so as to determine the effect of the state of the bacterium upon the lytic process. Since lysis is the result of the multiplication of the ultramicrobes the lytic process will not be complete unless all of the bacteria present in the suspension are capable of being attacked.

Instead of taking a suspension prepared from a young culture we may inoculate the bacteriophage into a fifteen-day old broth culture. A clearing of the medium, a partial lysis, results but a certain degree of turbidity remains. Nevertheless, it is possible to continue to use such a medium, making as many passages as may be desired. Some tube of the series when planted on agar or in bouillon will remain sterile, and a drop of this tube inoculated into a suspension of young bacilli will produce perfect lysis. In the old culture, then, the bacteriophage multiplies normally but does not produce lysis, or at least, the lysis is incomplete. What is the explanation of this reaction? To answer this it is sufficient to compare the results of counting the total number of bacilli existing in an old culture (this can be done by the method of counting cells) with the results secured by counting the viable organisms only (done by the plating method). For a confirmation of this type, a Shiga culture in Martin's bouillon is made, incubated for fourteen hours, and allowed to stand at laboratory temperature for fifteen days. The total count of bacillary bodies will be about 625 millions; that of the viable bacilli, that is, those capable of yielding colonies when transferred to agar, will be about two millions in each half cubic centimeter of culture. Now, as we have seen, the bacteriophage is only able to develop at the

expense of living bacteria, these being the ones which are lysed. In the old suspension which we have mentioned in which there is only about one organism in three hundred which is capable of being dissolved, it can readily be comprehended that if lysis of a suspension be taken as a criterion, the bacteriophage appears to be without action in such a culture.

It is useless to work with old cultures. In a broth culture of Shiga, after only twenty-four hours of incubation, as has been shown, about one-third of the organisms present are incapable of producing colonies when planted on agar. If, on the other hand, an agar slant culture is utilized, almost all of the bacteria are living after twenty-four hours at 37°C. A twenty-four hour bouillon culture will, then, remain slightly turbid when the lytic process is accomplished, while a suspension made in broth from a young agar culture containing the same number of bacteria will be perfectly limpid when the lysis is achieved. In this last case all of the bacteria were living and susceptible to the attack of the bacteriophage. It is for this reason that it is preferable to effect cultures of the bacteriophage in a suspension rather than directly into a bouillon culture.

Certain bacteria give a homogeneous growth in a young culture in bouillon but when taken from agar they can be suspended only with difficulty. *B. pestis* is such an organism. When working with such bacteria it is preferable to have the bacteriophage act on a broth culture in the following manner. A bouillon tube is lightly inoculated with the bacterium. When the culture has clouded, the bacteriophage active for this bacterial strain is introduced and at the same time the culture is diluted with an equal volume of sterile medium. This dilution should be made before the bacteriophage has had time to multiply sufficiently to parasitize an appreciable number of bacteria. Thus, the bacterial culture at the time of "departure," that is, when the bacteriophagous organisms are sufficiently abundant, will consist almost entirely of young bacilli, readily subject to attack.

It has been demonstrated that the products of bacterial growth as found in an old culture, products which, as is well-known, inhibit the development of bacteria (as in the so-called "vaccinated" media) are without effect upon the lytic phenomenon.

Experiment III. Cultures of *B. dysenteriae Shiga*, aged fifteen days and eighteen hours respectively, are centrifugalized. The sediment from the first culture is suspended in the supernatant fluid of the second, and the sediment of the second culture in the fluid of the first. Both suspensions thus formed are inoculated with a drop of a culture of the bacteriophage. The suspension consisting of "old" bacilli and "young" medium remains turbid; that of "young" bacilli and "old" medium becomes perfectly limpid after seven hours.

Thus, while the products of bacterial metabolism are not inhibitory for the lytic process, the products of lysis, as we will see, exert quite a different action. These products are the result of the activity of the ultramicroscopic bacteriophage, and as such, they impede its activity.

THE INFLUENCE OF THE MEDIUM

It is evident from the foregoing experiments that the true culture medium of the bacteriophage is the living bacterium. The nature of the fluid in which the bacteria are suspended is without direct influence upon the culture of the bacteriophage, provided only the bacteria remain living in it throughout a sufficient period of time and provided it does not alter the constitution of the bacterial cell. Experiment confirms this statement.

The only additional condition regarding the medium is that it be alkaline in reaction. Lysis will not take place in a medium of acid reaction.[6]

Experiment IV. Peptone water (containing 25 grams of Chassaing peptone and 5 grams of NaCl per liter) is neutralized to phenolphthalein. The medium is then frankly alkaline to litmus. It is then distributed into tubes, 10 cc. to each. Hydrochloric acid is added to each tube in dilutions to form an increasing scale of acidity. All of the tubes are inoculated with a concentrated suspension of Shiga, sufficient to yield a normal suspension of 250 millions per cubic centimeter. Finally, each tube is inoculated with 0.001 cc. of a culture of the bacteriophage. After twenty-four hours the numbers of bacteriophage in the several tubes are determined by the method previously described.

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[6] Certain commercial peptones contain glucose in appreciable quantity, hence their use may be attended by failure.

TUBE	REACTION TO PHENOLPHTHALEIN	APPEARANCE OF THE SUSPENSION AFTER TWENTY-FOUR HOURS	NUMBER OF ULTRAMICROBES PER CUBIC CENTIMETER
1	0	Very slight clouding	400,000,000
2	− 2	Very slight clouding	500,000,000
3	− 4	Limpid	500,000,000
4	− 6	Limpid	1,250,000,000
5	− 8	Limpid	2,750,000,000
6	−10	Limpid	1,000,000,000
7	−12	Limpid	1,000,000,000
8	−14	Slight turbidity	250,000,000
9	−16	Turbid	500,000
10	−18	Turbid	1,000,000
11	−20	Turbid	500,000
12	−22	Turbid	None

Neutrality to litmus corresponds to about − 16, thus it may be concluded that the bacteriophage ceases to grow when the medium presents even the slightest acidity. The ultramicrobial elements detected in the slightly acid tubes—those where lysis is not produced—is not an indication of multiplication. They are simply the ultramicrobes which were inoculated, still living. When the medium is decidedly acid even these elements are destroyed.

The following experiment demonstrates this still better and further confirms the fact that the bacteria constitute the only culture medium for the bacteriophage.[7]

[7] As will be shown in a later chapter, bacteria in general are destroyed after exposure for a very short time in physiological saline. On the other hand, the different strains of dysentery bacilli present a variable resistance. Certain races are no longer cultivable in bouillon after an exposure of five to six hours, others remain cultivable up to 45 to 50 days. Experiments involving lysis in physiological saline ought to be performed with strains of the last type. Moreover, it is necessary to employ an extremely active bacteriophage, capable of exerting its action in the minimum length of time—if possible, one capable of producing complete lysis in about three hours. A saline suspension of a bacterial strain of weak vitality may be already sterile after four to five hours, that is to say, the great majority of the bacteria are dead before the lytic process is able to be effected. And the bacteriophage is without action on dead bacteria.

Experiment V. One hundred cubic centimeters of a suspension of young Shiga bacilli are prepared in 0.85 per cent saline, neutral to litmus. This suspension is inoculated with five drops of an earlier saline culture of the bacteriophage, and the material is divided into five portions, 20 cc. in each tube. The first of these remains as already prepared. The second is rendered alkaline by the addition of NaOH in a quantity sufficient to give an alkalinity equivalent to 10 mgm. of NaOH per liter. The remaining tubes are also rendered alkaline, the third having a reaction equal to 25 mgm. of NaOH per liter, the fourth equal to 50 mgm., and the fifth equal to 100 mgm. After incubation for eighteen hours the first tube shows its original turbidity, tube 2 is cloudy, and tubes 3, 4, and 5 are almost perfectly clear. In these the turbidity is such that it can just be detected and is due to the fact that a certain number of the bacilli had died in the saline before they were attacked. When the lysis is completed in the last three tubes, to tube 1, which maintained its original turbidity, NaOH is added in an amount equal to 100 mgm. per liter. Twelve hours later lysis has taken place; the suspension has been transformed so that it possesses a transparency comparable to tube 5. It has been demonstrated that this degree of alkalinity by itself is without effect on the Shiga bacilli, since they develop normally in bouillon containing as much as 500 mgm. of soda per liter. From the cleared tubes all subcultures remain sterile.

The bacteriophage can be indefinitely cultured in series in a slightly alkaline saline solution. Indeed, the salt (NaCl) itself can be dispensed with. Serial cultures have been maintained in chemically pure water containing only 25 mgm. of soda per liter.

Certain experiments with a synthetic medium, capable of maintaining a culture of the Shiga bacillus, were performed and are not without interest. Growth of the dysentery bacilli and complete lysis by the bacteriophage are secured in a medium of the following composition: water, 80 cc.; sodium chloride, 1 cc.; potassium phosphate, 1 cc.; sodium phosphate, 1 cc.; and asparagine, 3 cc., all in 10 per cent solutions. The medium is rendered alkaline in accordance with the nature of the experiment to be performed.

In bouillon a relatively high alkalinity does not interfere with lysis.

Experiment VI. Five tubes, each containing 10 cc. of bouillon rendered alkaline to—8 are seeded with a suspension of the Shiga bacillus and then inoculated with 0.02 cc. of a culture of the bacteriophage. A N/10 solution of NaOH is added; to the second tube 0.5 cc., to the third 1 cc., to the fourth 1.5 cc., and to the last 2 cc. The first four tubes are perfectly lysed after eighteen hours, only the fifth remains clouded.

It may be affirmed, therefore, that, aside from the matter of alkalinity, the composition of the medium with respect to its nutritive properties, exerts no influence on the development of the bacteriophage. From the moment when it has at its disposition living and normal bacterial cells, against which it is active, it multiplies—at the expense of these bacteria which constitute its sole culture medium.

CULTURE OF THE BACTERIOPHAGE ON SOLID MEDIA: ISOLATED COLONIES

It has been stated that the bacteriophage shows the formation of obvious colonies on agar, and that in order to obtain them it is only necessary to inoculate a broth suspension of the Shiga organism very lightly with a culture of the bacteriophage and to distribute a drop of this suspension on agar. After incubation the covering of bacillary growth presents a number of areas free of all apparent culture. If the inoculation of the bacteriophage has been massive the surface of the agar appears sterile. Let us consider the characters of these cultures.

When the surface of the agar remains bare because of the large number of bacteriophagous organisms and maintains this appearance indefinitely it has become unsuited for the cultivation of the Shiga bacillus. When inoculated at such a time with a culture of this bacillus, even in a very abundant sowing, the slightest development cannot be detected. The medium is, however, normal for another bacterium. If inoculated with the cholera vibrio, for example, the growth will be as luxuriant as if planted upon fresh medium. Hence, if *B. dysenteriae Shiga* does not grow it is only because the bacteriophagous organisms remain on the surface of the agar and exercise their dissolving action on the bacteria deposited thereon. This is readily confirmed. If we take a tube of agar which has remained apparently sterile after having been inoculated with a suspension of the bacteria containing a bacteriophagous culture, and if the surface of the medium in such a tube is washed with a few drops of sterile bouillon and to this is added a fresh suspension of bacteria, this suspension will be lysed within a few hours.

It sometimes happens, especially when using agar somewhat dried out, that a few colonies of Shiga are obtained, always located at the extreme edge of the layer of agar. We will return to this extremely interesting particular in the discussion of secondary cultures.

If, instead of a continuous covering of the bacteriophagous growth the ultramicrobes are deposited in limited areas—and this is readily accomplished by placing drops of culture on the sterile surface of a tube of agar, or again, by drawing lines over the surface with a platinum loop dipped in the culture of bacteriophage, and after the tubes have remained inclined for a few hours in the incubator to secure drying—we find that the areas impregnated with the bacteriophagous culture remain free of Shiga bacilli, but that these organisms grow, on the contrary, perfectly well on the parts not covered by the bacteriophage.

When in the suspension planted upon agar the number of bacilli is infinitely great and the number of the ultramicrobes is sufficiently small, the bacteriophage culture as individual units is distributed over the surface of the agar, and under such circumstances the bacterial layer will appear studded with apparently sterile areas. These areas, or plaques, have a circular form with a diameter of from 1 to 5 mm. The plaques are in general of the greatest extent when the suspension is somewhat weak although sufficiently concentrated to give a continuous layer of growth rather than isolated colonies. On such a tube the areas are larger as the subjacent medium becomes thicker, that is, toward the bottom of the tube. Upon a Petri dish, where the agar layer is of essentially the same thickness throughout, all of the plaques of a given culture are of approximately the same diameter. As will be seen, the area of the plaque bears a relationship to the virulence of the bacteriophage which causes it.

If a tube or plate presenting plaques is held in the incubator at 37°C., or at an entirely different temperature, no change occurs in the plaques; their diameter remains indefinitely what it was at first. They are never covered or encroached upon by the bacterial culture. At no time does there exist within the extent of the plaque, whatever its size may be, microscopically visible bacterial cells. The plaque is always rigorously sterile.

As soon as the culture is well developed, as after 18 to 24 hours of incubation, if the centre of such a plaque is touched with a platinum wire and this is immersed in a culture of Shiga bacilli the bacteriophage develops in this suspension and the latter is lysed after a few hours. The plaque, although sterile, is not ultrasterile; it is in fact a colony of the bacteriophage.

Furthermore, if a trace of the bacillary growth at the periphery of a plaque is taken with a platinum wire and seeded on agar it remains sterile and inoculation into a bacterial culture shows that the bacteriophage is present there also. But when the bacillary layer is taken, not at the immediate edge of the area, but at a distance of two millimeters from it, for example, and planted, the tubes show the growth of a normal culture. The bacteriophage is not found.

If the culture showing the plaques is returned to the incubator and the tests are repeated three or four days later, that is, culturing the bacillary growth at a distance of two millimeters from a plaque onto agar and into a suspension it will be found that the bacteriophage is there present at that time. The bacteriophage has, therefore, gradually invaded the bacillary layer. This invasion is always slow—proceeding more and more slowly as time progresses—so that the ring invaded, even after several months, amounts to a zone but a few millimeters wide. Beyond the limits of this zone the Shiga organisms remain cultivable just as long as they do in a normal control culture without the bacteriophage.

The question immediately arises as to why the bacteriophage does not invade the entire layer of bacterial growth. For this there are two reasons. The bacteriophage attacks the bacterial cell most readily when the bacterium is young. When placed upon agar the bacteriophagous organisms find themselves located in the immediate vicinity of bacilli which reproduce actively as soon as they are deposited upon a nutrient medium. They find then, within their range, very young bacilli distributed in a very thin layer over the agar. Lysis is thus possible and the apparent sterility of the plaque results. But beyond this zone invaded by the bacteriophage during the first few hours the bacilli develop freely forming a layer of increasing thickness comprised of organisms of increasing age. In other words, a thicker and thicker

layer of bacilli always becoming more and more resistant to lysis develops. This can be readily demonstrated by direct experimental proof.

If the agar surface in a Petri dish is heavily seeded with a Shiga culture and at some point on this a drop of the culture of the bacteriophage is placed, and after a three-hour incubation period another drop of the bacteriophage is placed on the surface and this same process repeated after six, twelve and twenty hours, with continuous incubation of the plate during the intervals, it will be found fifteen hours later that the areas upon which the first three drops were placed have remained sterile—no bacillary growth has taken place. At the point where the fourth drop was placed, that is, after the culture had been incubated for twelve hours, there is a thin layer of growth composed of dead bacilli. The area where the drop of bacteriophage was placed after twenty hours presents an appearance practically normal. These five spots, then, represent the diverse aspects of an isolated colony of the bacteriophage, as from the centre to the periphery.

The second reason is of a more general nature, representing a phenomenon common to the majority of cultivable organisms. The colonies of the bacteriophage act absolutely like colonies of those bacteria which, except for organisms such as *B. proteus*, never progressively invade the surface of solid media. Thus, if the Shiga bacillus is inoculated upon agar in an amount suitable to yield isolated colonies, after 18 to 24 hours, each colony will be from two to four millimeters in diameter, the largest colonies to be found at the points where the medium has the greatest depth, that is, toward the bottom of the tube. Such colonies increase in size but very slowly, always more and more slowly as time progresses, and even after two months, the zone of increase will not be greater than a few millimeters. From the bacteriological point of view it is not peculiar, as has been suggested, that the bacteriophage does not invade the entire bacterial layer. It must be borne in mind that the bacteriophage, far from being dissimilar to other cultivable organisms, behaves, when in isolated colonies, exactly like a colony of bacteria.

Why does not the bacterial colony continue to increase in size and to invade the entire surface of the medium? Because the

soluble substances resulting from the vital activity of the bacteria diffuse into the agar and these substances constitute an actual specific antiseptic which limits the culture. The medium is "vaccinated" around the colony. The deeper the agar layer, or the farther the colonies are separated, the greater the volume of the substratum capable of diluting this antiseptic substance, and the larger will be the colony. The situation is precisely the same with the bacteriophage; the more scattered the colonies and the deeper the substratum, the greater the diameter. Direct experimentation proves the correctness of this interpretation, and that the soluble substances elaborated during the lytic process,—substances resulting from the vital activity of the bacteriophage,—inhibit the vital processes, delay growth, and prevent the accomplishment of total lysis.

EFFECT OF THE CONCENTRATION OF BACTERIA IN THE MEDIUM; INHIBITORY EFFECTS OF THE PRODUCTS OF LYSIS

In all of the experiments involving the action of the bacteriophage in a liquid medium which we have up to the present considered the bacterial suspension has contained approximately 250,000,000 organisms per cubic centimeter.

What occurs if the concentration of suspended bacilli is varied between 50 and 500 millions? The end result will always be the same—a complete lysis—and even the time required for this lysis will not greatly vary for the inoculation of a given quantity of the bacteriophagous culture, such is the rapidity of development of these organisms. Let us consider the two extreme cases.

Experiment VII (*A.*) The inoculation with the bacteriophage is massive, that is, 0.02 cc. In such a case the difference in time is most marked.

Number of bacilli per cc.	*Lysis is complete in*
50,000,000	4½ hours
100,000,000	4½ hours
200,000,000	5 hours
250,000,000	5 hours
300,000,000	5½ hours
400,000,000	6 hours
500,000,000	8 hours

(*B.*) The inoculation with the bacteriophage is very weak, in the following experiment, 0.00,000,1 cc.

Number of bacilli per cc.	*Lysis is complete in*
50,000,000	14½ hours
100,000,000	14½ hours
200,000,000	14½ hours
250,000,000	14½ hours
300,000,000	16 hours
400,000,000	16 hours
500,000,000	18 hours

These results are readily understood. In the first case the number of ultramicrobes is very great from the start, all of the bacteria, or by far the greater part of them, are immediately attacked, and this quickly arrests the development of the bacterial culture. In the second case, as a result of the small number of ultramicrobes inoculated, very few of the bacteria are at once attacked, and those which remain unharmed are free to develop, so much the more as the suspension is the more dilute. To be convinced of this it is only necessary to observe the suspensions and to compare their relative opacity from time to time. All become more and more turbid during the first few hours after the inoculation, and in five or six hours after the inoculation they all present a comparable opacity, corresponding to approximately 650,000,000 bacilli per cubic centimeter. In a word, whatever may be the original titre of the suspension at the time when it is inoculated with a limited number of the bacteriophagous organisms, the latter must always operate on a suspension of about 650,000,000 bacilli per cubic centimeter, since in all cases the bacteria reproduce until they attain this number. Hence, lysis will always take place within very nearly the same length of time.

A suspension of young bacilli, containing about 500,000,000 bacilli per cubic centimeter is completely lysed by the action of a bacteriophage of *maximum activity*. Beyond this figure the medium never entirely clears, and remains the more cloudy the more concentrated the suspension, regardless of the number of ultramicrobes inoculated. A suspension containing 1,000,000,000 bacilli per cubic centimeter, for example, will never be completely lysed, whether it is inoculated with a few individual bacteriophagous organisms or whether it is inoculated with some thousands of millions. However, in working with suspensions which are ex-

tremely heavy it is found that the bouillon transplants made on to agar after eighteen to twenty-four hours are always sterile. The bacilli have been killed but not completely lysed.

Experiment VIII

SUSPENSION OF BACILLI (MILLIONS PER CUBIC CENTIMETER)	INOCULATED WITH CULTURE OF BACTERIOPHAGE	ASPECT OF SUSPENSION AFTER TWENTY-FOUR HOURS
	cc.	
5,000	0.1	Turbid
2,000	0.1	Cloudy
1,000	0.1	Slightly cloudy
500	0.1	Clear

After incubation for 8 hours all cultures appeared the same.

The inhibitory force which interferes with lysis is due to the accumulation of the soluble products resulting from the lytic process, that is, to the activity of the ultramicroscopic bacteriophage itself. In this respect the action of the bacteriophage is in accord with a phenomenon common to all cultivable microorganisms.

Experiment IX. A bouillon suspension containing 250,000,000 bacilli per cubic centimeter is inoculated with 0.001 cc. of a culture of the bacteriophage. The next morning, or after fourteen hours, lysis is complete. A count of the bacteriophage shows that there are 1,600,000,000 per cubic centimeter. At this time a concentrated bacterial suspension is added to the lysed suspension in such concentration that the titre amounts to 250,000,000 per cubic centimeter. Seven hours later the medium is again limpid, and a count shows the presence of 2,100,000,000 ultramicrobes. This second lysis completed, the bacterial content is again restored. This time lysis is hardly accomplished in 48 hours, indeed, at this time the bouillon is not quite clear. A count gives 2,400,000,000 ultramicrobes per cubic centimeter. At this time, then, the medium contains in each cubic centimeter the dissolved substance of 750,000,000 bacilli. The suspension is made up to a concentration of 250,000,000 once more (for the fourth time). Eight hours later the culture has cleared somewhat but remains decidedly cloudy. The count shows 2,600,000,000 ultramicrobes. Inoculations upon agar and into broth remain sterile.

It is plainly to be seen, therefore, that the more concentrated the medium becomes in dissolved substances the more marked becomes the inhibition and the less active the culture of the bacteriophage.

The quantity of the bacteriophagous germs inoculated into the suspension is without influence on the final result.

Experiment X. The following tubes containing suspensions of the Shiga bacillus are prepared.

Tubes 1 and 5 contain 250 million per cubic centimeter
Tubes 2 and 6 contain 500 million per cubic centimeter
Tubes 3 and 7 contain 1000 million per cubic centimeter
Tubes 4 and 8 contain 2000 million per cubic centimeter

Tubes 1, 2, 3, and 4 are inoculated with 0.001 cc. of a culture of the bacteriophage. Tubes 5, 6, 7, and 8 are inoculated with 0.5 cc. of the same culture of bacteriophage, or, 500 times as much as in the first set. After eight hours tubes 1 and 5 are limpid. After fourteen hours tubes 1, 2, 5, and 6 are limpid. After four days tubes 1, 2, 5, and 6 are still limpid, tube 3 is very slightly cloudy, tubes 4 and 7 are cloudy, and tube 8 is turbid.

It is thus apparent that lysis is not affected by the number of the ultramicrobes inoculated. We will see in a subsequent chapter which treats of the resistance of the bacteria to the bacteriophage, that contrary to what one would *a priori* suppose, lysis of a culture is even more perfect when the amount of the bacteriophage added to the suspension is rather small.

Quite aside from the quantitative relationships, a suspension may vary in "quality." One may work with bacteria of different ages, or with organisms of different strains. In so far as difference in strains is concerned, the course of the phenomenon remains essentially the same—at least in so far as the Shiga bacillus is concerned. We shall see that it is not the same with certain other bacteria, *B. typhosus* for example.

With reference to the question of the age of the bacilli subjected to the action of the bacteriophage we have already seen that the younger the bacillus the more readily it is attacked. This difference is due solely to the state of the bacillus itself and not to the soluble substances resulting from its activity;—such substances as result in a "vaccination" of the medium, to use a common expression. The bacteria vaccinate the medium for themselves through the products of their activity. The bacteriophage does the same thing. But the soluble products resulting from their respective activities have nothing in common.

EFFECT OF EXTERNAL PHYSICAL CONDITIONS

The presence or absence of oxygen has no effect upon the course of the phenomenon. The rapidity of multiplication of the ultramicrobes and the duration of the lytic process are the same in aerobiosis as in anaerobiosis.

On the other hand, as would naturally be expected, the effect of the temperature is marked.

Experiment XI. Three tubes containing a suspension of the Shiga bacillus are inoculated, each tube receiving 0.00,000,01 cc. of the bacteriophage culture. These tubes are placed at different temperatures; the first at 8°, the second at 22°, and the third at 37°C.

The suspension held at 8°C. shows no lysis after twenty-four hours, but after 15 to 16 days lysis is complete. The number of ultramicrobial elements at this time is 180,000,000 per cc. as compared with 20 to 25 per cc. when they were introduced.

The suspension kept at 22°C. shows that multiplication commences after three hours. At this time the count is 75 ultramicrobes per cubic centimeter. After five hours the count is 8,000, after eight hours 190,000, and at twenty-five hours lysis is complete and the count is 780,000,000 per cubic centimeter.

The suspension held et 37°C. shows 210 ultramicrobes after two hours, 10,000 after three and one-half hours, 200,000 after five hours, and 1,700,000,000 per cubic centimeter after thirteen hours, with a complete lysis.

Between 37 and 41°C. the course of the reaction does not show appreciable variation. Between 41 and 44°C. the lysis is less and less complete. For this there are two reasons; the development of the ultramicrobes is less and less active, and the number of bacilli killed in consequence of the elevation of temperature is greater and greater. As a result the number of organisms capable of being attacked and dissolved are less and less numerous. However, serial cultivation of the bacteriophage at 44°C. is still possible. The ultramicrobe can be cultivated at temperatures higher than those supported by *B. dysenteriae*. With the latter growth ceases at 43°C.

In effect, the optimum temperature for the bacteriophage is the same as that for the bacterium, as is but logical, since all experimental work demonstrates that the more closely the bacterial cell approaches normal so much the better is it attacked.

EFFECT OF ANTISEPTICS UPON LYSIS

To complete the macroscopic study of the phenomenon it may be well to consider what effect different substances that may be added to the suspension may have upon the lytic process.

As we will see with reference to the properties of the bacteriophage, although it does not present a resistance as marked as some of the ultramicrobes to chemical and physical destructive agents, it is, nevertheless, less susceptible than the majority of cultivable organisms.

From the particular point of view of lysis, we must recall that the action of antiseptics is complex. The bacteriophage is only able to grow at the expense of living bacteria and all action exerted on the bacteria of a suspension are reflected in the phenomenon of lysis, even if these actions are weak or wholly lacking upon the bacteriophage itself.

The special resistance of the bacteriophage to antiseptics does not modify the lytic process.

Antiseptic substances may be introduced into the suspension in amounts sufficiently weak to render their effect on the bacteria negligible and thus fail to alter the course of the phenomenon. If, on the contrary, the amount of antiseptic is such that it is capable of recognition, the bacteriophage may be unable to multiply for lack of normal bacteria and lysis is prevented. In this last case it is easy to see that the conditions of the experiment are the same as if the bacteriophage was placed in the presence of bacteria previously modified by the antiseptic in question. And it has already been shown that under these conditions neither the growth of the bacteriophage nor the lysis resulting therefrom is accomplished.

The experiment previously presented showed that the bacteriophage failed to multiply in bouillon suspensions of Shiga containing one per cent of sodium fluoride, even though the bacteria remained alive during a period of time amply sufficient for complete lysis to be effected in the presence of normal bacteria. Glycerine acts in a different manner. In high concentrations this substance prevents the growth of the bacteria, but its activity is inhibitory rather than strictly antiseptic.

Experiment XII. Tubes of bouillon, containing glycerine in the following concentrations, are seeded with a drop of an eighteen-hour bouillon culture of *B. dysenteriae Shiga*. The results secured with the different concentrations are:

Bouillon + 5 per cent of glycerine: Very abundant growth
Bouillon + 10 per cent of glycerine: Weak growth
Bouillon + 15 per cent of glycerine: Very slight growth, with sediment
Bouillon + 20 per cent of glycerine: Clear medium, with abundant sediment of bacteria
Bouillon + 25 per cent of glycerine: Clear medium, slight sediment
Bouillon + 30 per cent of glycerine: Clear medium, slight sediment
Bouillon + 35 per cent of glycerine: Clear medium, trace of sediment
Bouillon + 40 per cent of glycerine: No growth whatever

All subcultures made from these tubes at the end of forty-eight hours give, in normal bouillon, normal growths.

B. typhosus is somewhat more sensitive to the action of glycerine. Even in a medium containing 10 per cent the growth is insignificant.

Bacteria suspended in glycerine bouillon, even in a concentration of 25 per cent, remain alive for at least forty-eight hours; that is, throughout a time amply sufficient for the bacteriophage to develop and to effect lysis, as was the case with the fluoride medium. But the following experiments show that the culture of the bacteriophage in the glycerine medium is absolutely normal while it is entirely lacking in the fluoride medium.

I emphasize the fact of the growth of the bacteriophage and the lysis which is the result of this growth in a glycerine medium, for it will be necessary to return to these experiments when we review the various proofs concerning the living nature of the bacteriophage.

Experiment XIII. Tube 1. Prepare a suspension of *B. dysenteriae Shiga*, 250,000,000 per cubic centimeter, in bouillon containing 35 per cent of glycerine. Inoculate with 0.02 cc. of the bacteriophage culture. Normal lysis occurs in eight hours. A control suspension of the Shiga bacilli in the glycerinated medium, but without the bacteriophage, yields positive subcultures up to the seventh day.

Tube 2. Shiga bacilli, 250,000,000 per cubic centimeter, are suspended in bouillon with 50 per cent glycerine. The medium is inoculated with 0.02 cc. of the bacteriophage. Normal lysis takes place in ten hours. The control suspension, without the bacteriophage, is cultivable up to the forty-eighth hour.

Tube 3. The Shiga bacilli, in the same concentration, are suspended in bouillon with 60 per cent of glycerine. Portions of this suspension are inoculated with various amounts of the bacteriophage culture, as follows:

a. With 0.5 cc. of the bacteriophage culture. With this amount lysis is complete in eight hours.

b. With 0.02 cc. of the bacteriophage culture. Complete lysis is obtained in nine hours.

c. With 0.0001 cc. of the bacteriophage culture. No lysis results. The ultramicrobes inoculated, too few in number, have not had time to develop before the bacilli are dead. A control suspension, without the bacteriophage, gave positive cultures only up to the 18th hour.

From this it appears that in glycerine broth the bacilli remain normally susceptible to attack by the bacteriophage just as long as they are living.

Although the bacteria die when suspended in the glycerine medium it can not be assumed that the glycerine acts as a true antiseptic, that is, that it modifies the bacterial protoplasm. No one would contend that sodium chloride in weak concentration is an antiseptic despite the fact that non-spore-forming bacteria suspended in normal saline survive for but a very short time: twenty-four to forty-eight hours in the case of the Shiga bacillus.

The experiments on lysis in the glycerinated media are, moreover, of great interest in that glycerine, in very high concentrations, sterilizes cultures of the bacteriophage.

Substances without action on the bacterial cells are, in general, without influence on lysis. This, for example, is the case with normal serum, ascitic fluid, urine, and 2.5 per cent sodium chloride. Calcium chloride, on the other hand, has a very marked inhibitory effect, and potassium chlorate delays lysis. In low concentrations magnesium sulfate and the phosphates of sodium and potassium favor lysis. This is particularly observed in the case of strains of the bacteriophage of feeble activity.

When sugars which are not fermented by the bacterium against which the bacteriophage is active are added to the suspension lysis is not modified. The addition of fermentable sugars is without effect if the inoculation of the bacteriophage is massive, but if the inoculation is weak lysis does not occur or remains incomplete, depending upon the amount inoculated.

The cause for these results is very obvious. The bacteriophage is very sensitive to acidity and with a minimal inoculation the bacteria begin to develop, to attack the sugar, and to render the medium acid before the ultramicrobes are present in adequate numbers to effect lysis in the time at their disposal.

SOLUBLE SUBSTANCES OF THE BACTERIA

Lysis of the bacteria by the bacteriophage is complete, without residue. In the last analysis this lysis can only be in the nature of a diastatic action. No bacterial activity is caused simply by the presence of the organisms as such, but rather by virtue of the secretory products which they elaborate. The ultramicrobes necessarily secrete some of these lytic diastases—the lysins—which liquefy the substances constituting the bacterial body. This point will be considered further. The chemical aspect of the reaction has been investigated but little since such studies naturally fall within the field of the chemist. All that may here be stated is that whatever may remain in solution in the clear medium when lysis is complete, it is not protein in nature, as is indicated by the reaction to heating.

The viscosity of a suspension containing 500,000,000 Shiga bacilli per cubic centimeter differs from that of the bouillon used in preparing the suspension. A volume of bouillon giving normally 100 drops gives but 97 when the suspension is lysed.

The decomposition of the substances derived from the bacterial bodies continues certainly for some time after the lysis. This can be shown for the Shiga bacteriolysate by the fact that if a rabbit is inoculated with the material immediately after the lysis is completed, it is killed by a dose essentially the same as the minimal lethal dose of the suspension. The toxicity of the bacteriolysate falls very rapidly; a week after the lysis it is markedly diminished, and in fifteen days the material is practically nontoxic. On the other hand, it is well known that the Shiga endotoxin is very stable. Obviously then, this endotoxin is rapidly destroyed by the bacteriophage. In a later chapter we shall see that the lysin secreted by the bacteriophage can be obtained by precipitation with alcohol.

THE ULTRAMICROBIAL BACTERIOPHAGE: AN ENDOPARASITE

We have already considered the mode of action of the bacteriophage from the point of view of its macroscopic characteristics. Various types of experiment allow us to penetrate somewhat further into the more intimate nature of the phenomenon.

Experiment has demonstrated that the bacteriophage is capable of development only at the expense of living bacteria, since these provide its sole culture medium. This cultivation apparently takes place within the interior of the bacterial cell, and ultramicroscopic observation shows that this is indeed the case. But first, let us consider some of the experiments which permit us to recognize the manner in which the infection of the bacteria is accomplished.

Attempts have been made to effect a culture of the bacteriophage of the Shiga bacillus in a filtrate derived from Shiga organisms grown in bouillon for various lengths of time—1, 7, 14, and 21 days—but all have failed. Two counts of the number of ultramicrobial elements, the one made immediately after the inoculation, the other after incubation for a week at 37°C., have given exactly the same number of germs. Thus, the bacteriophage is entirely incapable of multiplication in a medium containing only the secretory products of the bacterium. The bacterial body itself is essential.

If the bacteriophage actually proliferates within the interior of the bacterial cell, the ultramicrobes inoculated into a suspension ought, before all multiplication, to disappear from the fluid. In fact, each ultramicrobe ought first to penetrate a bacterial cell, to multiply there and to reappear in the fluid only when this cell is destroyed. It is easy to verify this hypothesis.

Experiment XIV. The following suspensions are prepared:

(1) 100 cc. of a suspension of the Shiga organisms containing 250,000,000 bacilli per cubic centimeter. This is inoculated with 0.25 cc. of a culture of the bacteriophage.

(2) 100 cc. of a suspension of the cholera vibrio, containing 250,000,000 per cubic centimeter. This also is inoculated with 0.25 cc. of the same culture of anti-Shiga bacteriophage.

(3) 100 cc. of bouillon containing only 0.25 cc. of the same bacteriophage.

The material of all three flasks is incubated at 37°C. Immediately after the inoculation, after thirty minutes, and again after 1 hour, 20 cc. are taken from each of the three flasks and centrifuged at 4,000 revolutions per minute for ten minutes.

There are thus nine tubes which have been centrifuged. From the supernatant fluid of each of these 0.02 cc. is taken and introduced into other tubes containing suspensions of the Shiga bacillus, and the counts of the ultramicrobe are made by plating 0.02 cc. of each of these nine tubes on six plates of medium. In this way an average of the counts can be obtained. The results of these counts indicate the number of ultramicrobes remaining in the medium, since those which have penetrated the bacterial cells before the centrifugation have been thrown down with the cells during this procedure and in consequence are to be found in the sediment.

The results of the counts are as follows:

Tube 1. Shiga suspension plus bacteriophage.

a. Counts of the material made immediately after the preparation are 214, 193, 187, 221, 229, and 183 plaques. The average is 204, representing 5,000,000 germs per cubic centimeter in the original suspension immediately after inoculation.

b. Counts on the suspension after incubation for 30 minutes are 3, 7, 4, 6, 6, and 3 plaques. The average is 5. This indicates that there are 125,000 bacteriophagous germs in the suspension thirty minutes after the inoculation. That is to say, of each 41 ultramicrobes inoculated 40 have disappeared.

A direct count of the suspension without centrifugation gives 5,000,000,000 elements per cubic centimeter. It is therefore certain that the ultramicrobes which have disappeared from the fluid during the centrifugation have gone down with the bacteria. And, as we will see in the two control experiments, in the absence of Shiga bacilli this sedimentation of the bacteriophage does not occur (at least, when centrifuged at a speed of 4000 revolutions).

c. After 1 hour, the count, made as before on the supernatant fluid gives an average of 8 plaques, or 200,000 ultramicrobes per cubic centimeter, a number essentially the same as that secured after thirty minutes. At this time a count of a suspension which has not been centrifuged gives 6,500,000, a number very close to that secured immediately after the inoculation.

It should be noted that in the hypothesis formulated with regard to intrabacterial growth, each colony forming within the interior of a bacterium gives rise to but a single plaque; just as in a bacterial count made by the same method a whole clump of bacteria seeded upon agar will yield only a single colony.

d. Counts made upon the suspension with and without centrifugation after one and one-quarter hours of incubation give the same number of ultramicrobes, about 90,000,000. The inoculated organisms have therefore increased from 5 to 90 millions; the increase being in a proportion of about

1:18. And this increase has taken place in apparently a very abrupt manner, only to be explained as a result of the liberation of actual colonies containing an average of about 18 germs. We will see by ultramicroscopic examination that the lysis of a parasitized bacterium takes place brusquely, by bursting.

Tube 2. Control. Suspension of *V. cholerae* plus the bacteriophage.

Counts made immediately after inoculation of the bacteriophage give: for the centrifuged material 201, for the non-centrifuged, 211 plaques.

After thirty minutes the counts are: for the centrifuged, 210; for the non-centrifuged, 216.

After one hour the counts are: for the centrifuged, 203; for the non-centrifuged, 199.

After one and one-half hours the non-centrifuged suspension gives 207.

Tube 3. Control. Sterile bouillon plus the bacteriophage. The counts immediately after the inoculation are: for the centrifuged, 206; for the non-centrifuged, 210.

After thirty minutes the corresponding counts are: 201 and 211.

After one hour, the counts are: 203 and 206.

After one and one-half hours the non-centrifuged medium contains 198.

As is evident, in the absence of bacteria capable of being attacked, nothing happens. The ultramicrobes remain inert in the liquid.

The nature of the multiplication taking place in the presence of the Shiga bacillus does not permit of any doubt on the following points.

1. After a contact of thirty minutes at 37°C. the ultramicrobes have almost entirely disappeared from the fluid; they are fixed by the bacteria. After one hour the situation is essentially the same.

2. After one and one-half hours there is an abrupt increase in the number of the bacteriophagous ultramicrobes.

3. The fixation is elective; it does not occur with *V. cholerae*, for example, for which the bacteriophage in question is without action.

From this it may be concluded that the culture of the ultramicrobes takes place within the interior of the bacillary body, and all the other observed facts support this conception based upon experiment. We can now understand the cause for the successive jumps noted in the culture of the bacteriophage, mentioned previously in connection with the multiplication of the germs. Each of the ultramicrobes inoculated penetrates to the interior of a bacillus and there multiplies up to the time when the bacillary

body bursts. This liberates the colony of ultramicrobes which have been formed in the bacterial protoplasm. A confirmation of this fact is obtained by examination of the phenomenon under the ultramicroscope, and by a study of the temporarily inhibitory action of an anti-bacteriophagous serum.

It has been shown that the successive increases in number are separated by intervals of approximately seventy-five to ninety minutes. On the other hand, a complementary experiment, conducted in the same fashion, but centrifuging the suspension at ten minute intervals during the first half hour, has shown that very few of the bacteriophagous germs are fixed during the first ten minutes, although they are almost all fixed after twenty minutes. The union, therefore, requires about a quarter of an hour.

Given the rapidity of multiplication of the ultramicrobe, and the time consumed in effecting each successive increment, it can readily be calculated that a single bacteriophage within a bacterium produces a colony varying in number from fifteen to twenty-five individuals; and it does this within the space of an hour or an hour and a quarter.

BACTERIOLYSIS UNDER THE MICROSCOPE

The anti-Shiga bacteriophage is always taken as an example in considering the action on dysentery bacilli.

It has been seen that when the inoculation with the bacteriophage is massive all the bacteria are attacked at the beginning, in other words, their multiplication is abruptly arrested. After two to three hours the medium commences to clear little by little and becomes completely limpid a short time later. If, on the contrary, the inoculation is minimal, the few ultramicrobes inoculated only affect an equal number of bacteria. The great majority remain unaffected and multiply as they would in a normal medium. But the ultramicrobes likewise multiply, following a progression more rapid than that pursued by the bacteria, so that within a few hours their number becomes equal to, or greater than, that of the bacteria. This is the time when macroscopic lysis commences.

1. Let us consider the first case, that of the massive inoculation. If we take from time to time a drop of the suspension

up to the point when lysis is complete, spread these drops on slides and stain, either with the Gram stain, with carbol-thionin, or by the Romanowsky-Giemsa method (all staining methods give essentially the same picture), results such as the following are secured.

A suspension of Shiga bacilli, 250,000,000 per cubic centimeter, is inoculated with 0.1 cc. of a culture of the bacteriophage and incubated at 37°C.

After fifteen minutes it appears as a culture of normal bacilli.

After thirty minutes it appears essentially the same, except that a few of the bacilli are poorly stained.

After forty-five minutes about 10 per cent of the organisms stain poorly.

Between one and two hours, the number of bacilli which stain badly continues to increase, and after 2 hours only a rare cell can be found which has taken the stain normally. At the same time, amorphous debris and granulations, derived most certainly from the bacteria already lysed, are seen. Similar material is seen very abundantly in old normal cultures of the Shiga bacillus. These granulations dissolve more slowly than the remaining portions of the bacterial protoplasm. Finally, and this is a most important point, spherical forms, more or less ellipsoidal, of variable dimension, always rare, measuring 4 to 7 by 3 to 5 μ may be detected. We will see in a moment to what they are due. There are occasional bacillary forms, well-stained, having a length of from 8 to 12 μ.

Between the second and third hours the amorphous debris considerably augments and the bacillary forms rapidly disappear. A few spherical forms are still to be seen.

After four hours lysis becomes more and more complete. Only a single poorly stained bacillus will be found in two or three fields.

Gradually the formless debris disappears, and, in turn, the granules. After thirty-six hours nothing whatever can be distinguished in stained preparations.

With the ultramicroscope at no time can there be seen elements other than the bacilli (whose number gradually diminish, to disappear entirely in about two hours) and the extremely fine granules. It can hardly be said that the latter represent formed elements. At the beginning the bacilli present a normal appearance. After forty-five to sixty minutes fine granules are seen, ever becoming more and more abundant, within the interior of the bacterial cells. The number of bacterial cells containing granules also rapidly increases with a corresponding diminution in the number of normal bacilli. The most interesting part of the observation[8] is that within one and one-quarter to one and

[8] First noted by P. Jeantet.

one-half hours after the beginning of the process the bacilli begin to swell, and the spherical bodies, containing a variable number of granules (averaging from 15 to 20) are, in comparison with a normal bacillus, from 3 to 5 μ in diameter. If the spherical bodies are observed with care it is seen that after a variable length of time, sometimes amounting to only about ten minutes, an actual bursting takes place, consuming but a fraction of a second. Immediately afterward, in the place of the spherical body there remains a slight cloudy floccule that slowly dissolves, thus liberating the fine granules. These spherical bodies are particularly abundant at the time when the lytic process is at its maximum rate. There can be no question concerning the nature of these bodies; they are bacilli which, operated upon by a force which can only be internal, take at first a globoid form and then rupture. This is the more certain since at times one can witness the rupture of swollen bacilli, even before they have assumed a spherical contour. This observation provides direct proof that the ultramicrobe develops and exerts its action within the bacterial cell. Destruction of the bacilli would be an entirely different process if the dissolving action were exerted on the exterior. The spherical form and the bursting prove beyond possible contradiction that the operating force is internal.

What do the fine granules that can be seen under the ultramicroscope represent? While nothing can be affirmed with absolute assurance there is nothing to preclude the supposition that they represent the ultramicrobes, basing this upon the comparative examination of cultures in which the number of ultramicrobes has previously been counted by plating upon agar. By such a procedure it is found that in taking two cultures presenting a great difference in count, a parallelism is always to be noted between the counts and the number of granules observed.

It would likewise be well to recall what we have already seen with reference to the multiplication of the germs, that this multiplication appears to take place in successive jumps (which correspond to the rupture of a large number of parasitized bacilli) and in which the number of ultramicrobes liberated after one and one-quarter to one and one-half hours corresponds to about eighteen germs to each single one inoculated. And we will see

that the number of granules consequent upon the rupture of a cell amounts to between 15 and 25. There is, therefore, a great probability that the granules are actually the ultramicroscopic bacteriophagous organisms.

2. We may consider the second case, that of a minimal inoculation. In this case the medium becomes more and more turbid before lysis actually commences.

A suspension of Shiga bacilli, containing 250,000,000 per cubic centimeter is inoculated with 0.0001 cc. of a culture of the bacteriophage, a very active strain being selected.

After 30 minutes the medium has its original turbidity; essentially that of a normal culture of the Shiga bacillus.

After one hour the original turbidity is still maintained. When smeared and stained all the bacilli are of normal shape, but an occasional form stains poorly.

After two hours the culture is about twice as turbid as at first. There is amorphous debris in the bottom of the tube. All of the bacilli appear to stain as normally. Many of the bacilli (about two in every three) are about four times the normal length, that is, of the bacilli used to seed the culture, and there are all intermediary forms. Oval and spherical forms are relatively numerous, but they are always fewer than would be expected from a comparative ultramicroscopic examination. These forms are indeed very fragile and are particularly liable to destruction during fixation upon the slide so that their demonstration in stained preparations requires great care.

After three hours the suspension is slightly cloudy. The bottom of the tube is covered with fine debris without definite form, with, from place to place, great amorphous masses and numerous granules resembling those encountered in very old cultures of normally grown Shiga bacilli. Only a single spherical form can be detected in a ten-minute search. Each field may contain a dozen large bacilli, well stained.

After four hours the turbidity is very slight. There is somewhat less material in the bottom of the tube, and this shows only a single poorly stained bacillus to a field.

After six hours the medium is limpid. There is still less deposit in the bottom of the tube and it is with difficulty that a single poorly stained bacillus may be found in searching 25 fields.

After eighteen hours nothing at all can be seen in the preparation.

As is evident, the aspect of this preparation differs but little from that seen in the former case, the only departure being that the bacilli which have grown immediately after inoculation, before the action of the bacteriophage becomes operative, present abnormally large forms.

A comparable ultramicroscopic examination in the two cases shows that in the last, where the inoculation was made with a bacteriophage which was extremely active, at the time when lysis occurs with greatest intensity, that is, between two and three hours after the inoculation, the spherical forms were present in greatest numbers. There were as many as two to three to a field, and their rupture was readily observed. When lysis is once terminated the most careful search fails to reveal such forms.

It is here fitting to recall an observation already made which should be noted by those wishing to investigate the subject. When a simple diastatic action is operative it proceeds with uniform rhythm when under identical conditions. This is not the case here. Up to the present time more than a hundred different strains of the anti-Shiga bacteriophage have been isolated and no two of them have been found to conduct themselves in an exactly identical manner. The final result is always as has been indicated, the phases of the phenomenon always progress in the same order, but the time of the reaction will vary. With one strain of the bacteriophage complete lysis is obtained in three hours, with another, only after twelve hours. The phases follow each other in one case four times more quickly than in the other.

Another point which should be remembered is that all that which has been said up to the present time has been in reference to bacteriophagous strains which were extremely active; that is to say, strains capable of producing complete lysis.

A summary of the foregoing shows that, in so far as the microscopic observations are concerned, there is no time when one can distinguish in stained preparations, whatever the magnification, microörganisms other than *B. dysenteriae Shiga*. Aside from the bacilli one can see only formless cellular debris becoming more and more abundant with the more complete destruction of the bacteria, the debris later dissolving gradually. Ultramicroscopic examination indicates that the ultramicroscopic bacteriophagous germs multiply within the interior of the bacilli, and this observation is corroborated by all experiments. Such examination also indicates that very probably the bacteriophagous elements are represented by the very fine granules which can be observed, first in the interior of the bacilli, and later in the ambient fluid.

CHAPTER II

The Bacteriophage and the Bacterium

Virulence of the Bacteriophage. Measure of Virulence. Resistance of the Bacterium. Secondary Cultures. Instability of Mixed Cultures. Characteristics of Mixed Cultures. Resistant Bacteria. Acquisition of Resistance. Production of Anti-lysins by the Bacteria. Multiple Cultures.

VIRULENCE OF THE BACTERIOPHAGE

In the preceding chapter we have taken note of the complexity of the phenomenon under study; a complexity resulting from the fact that there are simultaneously three elements which react, the one upon the others,—the medium, the bacteriophage, and the bacterium. Moreover, up to the present we have considered only the simplest case that may be presented, a bacteriophage of maximum virulence before which the bacteria always succumb. Often, the issue is very different, and for two reasons. The activity of the bacteriophage is not fixed, it varies along a scale extending from an action barely capable of detection to a lytic power most intense. The bacterium on its part is not passive; it defends itself.

We have already stated that the activity of the bacteriophage is a true virulence, in the exact meaning of the word, "the ability which a micro-organism possesses to develop within the body of a host and there to secrete toxic substances." Just as for each pathogenic bacterial type there is a scale of virulence, so also for each strain of bacteriophage there is a certain virulence. It is possible to exalt or to attenuate the virulence of a given bacterium and the same can be accomplished with the bacteriophage. Finally, just as higher forms when parasitized by a bacterium defend themselves and are capable of acquiring an immunity to this bacterium, so in like manner the bacterium attacked by a bacteriophage does not remain passive, but struggles, and may either be destroyed or acquire an immunity. All the vicissitudes of a conflict between an animal and an attacking

bacterium are duplicated in the struggle between the parasitic bacteriophage and the attacked bacterium. The resemblance is complete. It is only a matter of descending a degree in the order of size in the beings concerned.

It is also a property of living beings to never be the same at any two moments of their existence. If the phenomenon of serial transmissible bacteriolysis which we are considering were of purely diastatic nature, the activities as they unfolded would follow a fixed plan; for if the active element was not varied in quantity its quality would in all cases be constant. But we have seen that quite the contrary is the case. The phenomenon is independent of the quantity of the active element employed. The dominating feature is the quality of this element. Such are precisely the characteristics of vital activities. A poison acts in accord with its mass; a bacterium, with its virulence.

Experiment has already shown that a bacteriophage but weakly capable of attacking an organism is susceptible to increase in potency through successive passages in contact with the bacterium which it attacks. In order to recognize the differences presented between different strains of the bacteriophage it is preferable to work with strains freshly isolated from the organism.

Experiment XV. (A). Ten cubic centimeters of a suspension of Shiga bacilli are inoculated with 1 cc. of a filtrate made directly from the feces of a patient with dysentery. The suspension is held at 37°C. Counts of the ultramicrobes, made at different times during the incubation, give the following results when 0.01 cc. is plated on agar.

When plated immediately, there develop 16 plaques, representing 1,600 ultramicrobes per cubic centimeter. The filtrate from the feces therefore contained 16,000 per cubic centimeter.

After one and one-quarter hours, the count is 40 plaques, or 4,000 per cc.

After two and one-half hours, a 1:10 dilution gives 42 plaques, or 42,000 per cubic centimeter.

After three and three-quarter hours, a 1:100 dilution gives 18, or 180,000 per cubic centimeter.

After five hours, a 1:1000 dilution gives 4, or 400,000 per cubic centimeter.

After fourteen hours, the lysis is not complete, the medium is cloudy and becomes more and more turbid, so that after forty-eight hours it is very turbid. Here there is an abundant culture, but lysis is never complete. The bacteria have, then, acquired a certain resistance which has allowed them to reproduce in spite of the presence of the bacteriophage.

A result of this kind is usual when the filtrate is prepared from a stool taken shortly before the manifestations of convalescence appear.

(B) Ten cubic centimeters of the Shiga suspension are inoculated with 1 cc. of the filtrate prepared from the feces from the same dysentery patient, but collected 24 hours later, the patient now being convalescent. Counts of this mixture give:

When plated immediately, no plaques, or less than 100 ultramicrobes per cubic centimeter. Thus, the filtrate contained less than 1,000 per cc.

After one and one-quarter hours the plating shows no plaques.

After two and one-half hours there are 9 plaques, or 900 ultramicrobes per cubic centimeter.

After three and three-quarter hours, in a 1:10 dilution, there are 27 plaques, or 27,000 per cubic centimeter.

After five hours, a 1:1000 dilution shows 13 plaques, representing 1,300,000 per cubic centimeter.

In this last experiment (B) the ultramicrobes were present in the filtrate in very small numbers, certainly less than 1000 per cubic centimeter, that is, there were less than one-sixteenth as many as in the filtrate of the first preparation (A). Nevertheless, the suspension was completely lysed in ten hours and the fluid remained sterile indefinitely.

It should be noted that the multiplication of the ultramicrobes was much more rapid in the second experiment than in the first, and that this corresponds exactly with the idea of a greater virulence. These experiments show also that the number of ultramicrobes inoculated is without effect upon the intensity of the phenomenon, but that the important thing is the quality of the bacteriophage, that is, its virulence.

It is significant that the two strains of bacteriophage under discussion were derived from the same patient, but were taken at an interval of twenty-four hours. It is the same bacteriophage whose virulence has been increased *in vivo*.

It would be possible to cite a great number of experiments of the same order. On each page of this text facts will be found that show that the essence of the phenomenon is the virulence of the bacteriophage, a virulence extremely variable, exalted, or attenuated, or indeed absent for a given bacterium according to the conditions at the moment obtaining. This extreme variability observed especially *in vivo* is due to a variety of conditions. It is less *in vitro*, where we are able, within certain limits, to secure a relative stability.

EVALUATION OF THE DEGREE OF VIRULENCE

As will be shown in Part II of this monograph, it is necessary to the study of the processes of immunity associated with the presence of the bacteriophage, to be able to measure as exactly as possible the degree of virulence possessed by each strain of the ultramicrobe. The intensity of the action upon a bacterial suspension, or on a culture, in a liquid medium gives an indication of the virulence. A strain of maximum activity causes complete lysis in a relatively short period of time, varying between three and thirty hours. A less active strain causes only a partial lysis. This method of evaluation is, however, very crude and subcultures upon agar provide more precise determinations, particularly when dealing with strains which are but slightly active.

If we introduce into a bacterial suspension a drop of a filtrate containing a bacteriophage active for the bacterium in the suspension, and if we plate upon agar a drop of this material after variable periods of incubation, it is possible to follow the multiplication of the ultramicrobes. It is only necessary to count the isolated colonies, which assume, as we have seen, the form of circular plaques. If working with two or more strains of the bacteriophage, it is thus easy to follow the relative rapidity of their multiplication, and by the same fact, to measure their respective powers of growth at the expense of the bacteria parasitized, that is to say, their virulence.

The extension of the plaques furnishes a second measure of the rapidity of the multiplication of the ultramicrobes. Each plaque, representing a colony, results from the extension into the culture of the descendants of a single ultramicrobial element, deposited during the plating, at the expense of the bacteria in its environment. The more rapid the multiplication of the bacteriophage the more rapid the extension of the plaque. Thus, the diameter of the plaque permits a valuation of the degree of virulence of the bacteriophage which produces it.

Experiment XVI. The relative virulence of four strains of an antityphoid bacteriophage taken from a single patient convalescent from typhoid fever (Jeanne Del....) at different periods during this convalescence is determined.

A suspension of *B. typhosus* containing 250,000,000 bacilli per cubic centimeter is prepared and distributed into four sterile tubes, 10 cc. to the tube. Each of these tubes is then inoculated with 0.0001 cc. of a filtrate; the first with a filtrate prepared on the first day, tube 2 with that prepared on the second day, etc. After shaking, 0.1 cc. is taken from each tube and plated, as usual, on an agar plate, taking care that the agar layer in all the plates is of the same depth simply to make all the conditions comparable. After incubation the following results are obtained:

1. The filtrate prepared from the stool taken on the first day of the appearance of the bacteriophage shows 16 very small plaques, pin-point in size.

2. The filtrate derived from the stool of the second day after the appearance of the bacteriophage shows 31 plaques, having a diameter of less than 1 mm. each.

3. The filtrate made from the stool of the third day gives 52 plaques, with diameters of about 2 mm.

4. The filtrate prepared from the stool of the fourteenth day shows 42 plaques, each with a diameter of less than 1 mm.

Strain 3 is by far the most virulent, a conclusion that is supported by the fact that in its isolation it induced a total lysis of the bouillon culture of the typhoid bacillus. Strains 2 and 4 are much less virulent. Only by a dozen passages was it possible to effect an enhancement in virulence sufficient to give the same result. And at that time, when plated on agar the plaques had a diameter of 2 mm.

These experiments demonstrate very well that with equal virulence the plaques on the surface of a medium are approximately equal in size. The process of measuring virulence by counting the plaques and thus determining the rate of multiplication is certainly more exact than is observation of lysis in a fluid medium. It is, unfortunately, too complicated to be applied in routine practice when a large number of strains must be examined, as is the case when working with patients.

RESISTANCE OF THE BACTERIUM

In the first of the two experiments just cited (Experiment XV, A) we have seen that the bacterium was successful in developing in spite of the presence of the bacteriophage. The virulence of the bacteriophage, then, although constituting a most important factor in the phenomenon is not the only consideration. The bacterium is capable of resistance.

Up to the present time we have considered only the case where lysis was complete and permanent, and it has been specifically stated that the phenomenon assumes this form only when the bacteriophage possesses a maximum virulence and acts upon a limited quantity of suspension—10 to 20 cc. In spite of the fulfillment of these conditions it sometimes happens that a suspension which has been lysed in a normal manner with a perfectly limpid appearance, will some days later become turbid. Microscopic examination shows that the turbidity is due to multiplication of the bacteria, and tests of the biologic activity prove that this culture is composed solely of bacteria of the same species as was used in preparing the suspension upon which the bacteriophage was acting. According to the virulence of the strain of the bacteriophage being tested the number of tubes in which this reaction takes place, that is, the development of this secondary culture,[1] is more or less great.

Inoculations on agar or in bouillon of lysed suspensions, in which secondary cultures later develop, remain sterile up to the time that the secondary culture is formed. This does not often occur until five or six days after the lysis, sometimes even later.

Experiment XVII. A suspension of Shiga bacilli, containing 250,000,000 bacilli per cubic centimeter, is inoculated with 0.001 cc. of a culture of the bacteriophage. Normal lysis takes place in five hours, with the medium perfectly limpid. The lysed suspension is planted on agar and in bouillon 1, 2, 3, 4, 5, 6, and 7 days after the lysis is completed. All the plantings remain sterile. On the eighth day the lysed suspension is slightly clouded. On the ninth day a drop is inoculated into broth and on to three tubes of agar. Two of the agar tubes remain sterile, the third shows four small colonies. The broth tubes give a culture agglutinated in the sediment.

The resistance of diverse strains of a single bacterial species is not constant. Each strain appears, on leaving the organism, to be possessed of an individuality which is rapidly effaced by successive cultivations upon an artificial medium.

[1] In order to facilitate exposition I have called a "secondary culture" one growing again in a lysed suspension; a "mixed culture," the inoculation into a nutritive medium of a "secondary culture" with the coexistence in the medium of bacteria and bacteriophage.

In the following experiment the most powerful strain of the bacteriophage yet isolated is made to act upon two different races of the Shiga bacillus. One of these bacterial strains has been for a long time under artificial cultivation, being used by the Pasteur Institute for the inoculation of horses in the production of anti-dysentery serum (type strain). The other was recently isolated from the stool of a patient with dysentery (strain Jerv.).

Experiment XVIII. (*A*) Twelve tubes of the suspension of the type strain of the Shiga bacillus are each inoculated with 0.001 cc. of a culture of the bacteriophage. This latter has been carried on for a great number of generations always at the expense of a single bacillary strain. In all twelve tubes lysis is perfect, with complete clearing in four hours. After three days at 37°C. one of the tubes is slightly cloudy, the others are clear. (Five other experiments, each consisting of 12 tubes, with the same strain of bacteriophage and the same bacillus give the following results:—tubes showing secondary cultures in each set, 0, 2, 0, 3 and 1. There develop, then, 7 secondary cultures in the 60 tubes, or 12 per cent.)

(*B*) Twelve tubes of suspension were prepared with the strain Jerv., a strain with which the bacteriophage in question had never been in contact. Each of these tubes is inoculated with 0.001 cc. of the same culture of bacteriophage as that used in the preceding experiment (A). Seven of the 12 tubes give secondary cultures. The results from five other experiments with the same strains are, 9, 5, 10, 5, and 6 secondary cultures, or 70 per cent. A week later 12 cultures of the Jerv. bacillus are inoculated from one of the previous tubes that had remained clear. From these, 5 secondary cultures are secured.

A further passage made after another week, gives 4 secondary cultures in the 12 suspensions. After another week, a fourth passage, still taking the bacteriophage from a perfectly limpid culture, yields but one secondary culture among the twelve inoculated.

(*C*) At the beginning of convalescence in the dysentery case (Jerv.) a bacteriophage was isolated which was tested in the same manner both on the type Shiga strain and on the Jerv. strain. This last was derived from the patient early in the infection at a time when the intestinal bacteriophage had manifested no activity for this organism.

With the bacteriophage Jerv. on the type bacillus 4 secondary cultures develop among the 12 suspensions lysed.

With the bacteriophage Jerv. on the bacillus Jerv., there are no secondary cultures among the 12 tubes lysed. When repeated upon an additional 12 suspensions a single secondary culture develops.

It is then clear that the anti-Shiga bacteriophage is not equally active for all strains of *B. dysenteriae Shiga*. This fact is even more in evidence with other bacterial species, for example, with

B. typhosus, *B. coli*, *B. proteus*, and *B. pestis*. Each bacterial strain possesses an individual resistance, particularly when freshly isolated, which renders it more or less resistant to a bacteriophage accustomed to an *in vitro* existence. Later we will see that this resistance increases by a phenomenon of natural selection.

All of the phenomena in which the bacteriophage is involved, whether taking place *in vitro* or *in vivo* (the first are only an artificial reproduction of the last) are dominated by two factors,—the virulence of the bacteriophage and the resistance of the bacterium.

THE ORIGIN OF SECONDARY CULTURES

What is the intimate mechanism of the process that results in the formation of secondary cultures? *A priori* two hypotheses can be formulated. Two factors are present, a bacteriophage whose virulence may be attenuated, and a bacterium whose resistance may be augmented. Thus, are secondary cultures due to a weakening of the activity of the bacteriophage, or, do there exist in the bacterial suspension certain individual cells which acquire an immunity to the bacteriophage, thus leading to the development of a resistant race? The following experiments clearly settle the question in favor of the last hypothesis.

In the chapter treating of the isolation of the bacteriophage we have seen that in the large majority of cases the strains which are freshly isolated are of too low activity to effect a complete lysis of a bacterial suspension; cases where the presence of the ultramicrobe could only be detected by the presence of plaques upon the agar slants. These same strains were able to acquire, by successive passages, a very high activity, a potency which enabled them to bring about lysis of very heavy suspensions. This method of serial passages of the bacteriophage, in which it is forced to develop *in vitro* at the expense of a given bacterium, corresponds exactly with the method of Pasteur for effecting an enhancement in virulence of a bacterial race by repeated passage through a given animal species.

This single experiment, repeated a considerable number of times,—in fact, each time that a bacteriophage of low virulence is isolated from the body—shows that secondary cultures are not produced by a simple diminution in the virulence of the bacterio-

phage. Indeed, there is, on the contrary, an enhancement with each passage, even if macroscopic lysis is not to be seen. For this the following experiment offers direct proof:

Experiment XIX. The contents of a tube that gave a secondary culture (as described on page 72) is filtered through infusorial earth and a bougie. Twelve tubes of a Shiga suspension are inoculated, each receiving 0.001 cc. of the filtrate. Perfect lysis is seen in all tubes, and in all but one the lysis is permanent. This single tube again becomes turbid after 4 days.

From this it is clear that the bacteriophage has not lost in virulence, and that secondary cultures can not be ascribed to a change in that direction. The bacteriophage remains virulent, coexisting with bacteria which have become resistant. The secondary cultures, then, are the result of an adaptation undergone by the bacterium which acquires an immunity to its parasite.

It has already been shown that the number of ultramicrobes inoculated is without influence on the appearance of secondary cultures. The conflict is not one of numbers; it is rather a struggle in which the significant factors are virulence on one side and ability to resist on the other.

Experiment XX. A suspension of *B. dysenteriae*, 250,000,000 per cubic centimeter, is distributed into 6 tubes and these are inoculated with variable quantities of the same bacteriophage culture. The following results are obtained:

TUBE	AMOUNT OF BACTERIOPHAGE CULTURE INOCULATED	RESULTS
	cc.	
1	0.1	Normal lysis, secondary cultures
2	0.02	Normal lysis, no secondary cultures
3	0.004	Normal lysis, no secondary cultures
4	0.002	Normal lysis, secondary cultures
5	0.0002	Normal lysis, no secondary cultures
6	0.00002	Normal lysis, no secondary cultures

The tubes yielding secondary cultures are distributed at random throughout the series, showing no fixed relationship to those tubes in which lysis was permanent.

Experiment XXI. This experiment shows the serial activity of the bacteriophage together with the appearance of secondary cultures. Each tube of the series is prepared with a suspension of *B. dysenteriae*, 250,000,000 per cubic centimeter, and into each is introduced 0.001 cc. of the lysed suspension of the preceding tube. Transfers are made after twenty-four hours, that is, at a time when lysis is complete.

DATE	A FRESH SUSPENSION RECEIVED THE MATERIAL INDICATED	RESULT
July 8	0.001 cc. of bacteriophage culture	Permanent lysis
July 9	0.001 cc. of suspension lysed on July 8	Permanent lysis
July 10	0.001 cc. of suspension lysed on July 9	Secondary cultures in 3 days
July 11	0.001 cc. of suspension lysed on July 10	Permanent lysis
July 12	0.001 cc. of suspension lysed on July 11	Permanent lysis
July 13	0.001 cc. of suspension lysed on July 12	Permanent lysis
July 14	0.001 cc. of suspension lysed on July 13	Secondary cultures in 4 days
July 15	0.001 cc. of suspension lysed on July 14	Permanent lysis
July 16	0.001 cc. of suspension lysed on July 15	Permanent lysis
July 17	0.001 cc. of suspension lysed on July 16	Permanent lysis

Certain salts, when added to the suspension in very minute quantities, 0.1 mgm. to 10 cc. of culture, favor the development of secondary cultures. The salts of lead (nitrate and acetate) and of silver (nitrate and sulfate) act in this way. The soluble phosphates and magnesium sulfate appear to be without action. With a single strain of bacteriophage and a given strain of bacillus the development of secondary cultures is, in general, more frequent when the suspension is prepared from agar cultures several days old than when made from fresh cultures.

At first thought it appears strange that when secondary cultures develop with a strain of bacteriophage of high potency, they appear in some tubes and not in others. The following experiment offers an explanation for this.

Experiment XXII. Two flasks, each containing 200 cc. of a *B. dysenteriae* suspension (250,000,000 per cubic centimeter) are inoculated with 0.04 cc. of a culture of the bacteriophage (the same strain as that used in the preceding experiments). Immediately after inoculation the contents of the first flask is distributed into 20 tubes, 10 cc. to each. In all of these lysis takes place normally, being permanent in 19, showing a secondary

culture in 1. The second flask is portioned out the next day, that is, after lysis is completed, 10 cc. being placed in each of 20 tubes. None of these become turbid. When this second part of the experiment is repeated, 18 remain clear, and 2 tubes yield secondary cultures.

Each flask of suspension contained 50,000 million bacilli, and the above experiments show that of this number but one or two were capable of acquiring an immunity to the very active bacteriophage. It is these "immune" bacilli which give rise to organisms that enjoy the same degree of resistance.

Secondary cultures, then, have their origin in the operation of the phenomenon of natural selection, whereby some bacilli show a greater aptitude than others to the acquisition of a resistance to the bacteriophage.

The phenomenon of secondary culture formation is governed by the individual properties of the two admixed organisms,—bacterium and bacteriophage. Against a single strain of bacterium the less virulent the bacteriophage the greater will be the proportion of secondary cultures, or, in other words, the greater is the number of bacilli in the suspension capable of acquiring a resistance.

Against a given strain of bacteriophage the different strains of a single bacterial species are not endowed with an equal resistance. With certain strains secondary cultures will be the rule, with others, the exception, and with still others, they will never occur.

We will shortly see the reasons for this variation; at present we may say that the degree of resistance possessed by a bacterium to a bacteriophage is, for a given bacterial species, in direct relation to the degree of virulence which this bacterial strain possesses for the higher organism which it is capable of invading.

INSTABILITY OF MIXED CULTURES

Mixed cultures result from a state of equilibrium between the virulence of the bacteriophage and the resistance of the bacterium. But these two factors are by nature variable and vary in intensity from one time to another, being influenced by the circumstances of the moment. This equilibrium can be interrupted experimentally in either direction, so as to favor either the one or the other of the factors.

For example, if we place a small quantity of a secondary culture in normal saline, or preferably in 30 per cent glycerine bouillon, that is to say, in a medium which interferes with the reproduction of the bacteria and which exerts no destructive action on the bacteriophage, the equilibrium is disturbed in favor of the bacteriophage.

The ultramicrobe is very sensitive to the action of acids, and if transfers from a secondary culture are made upon glucose agar it is found that the bacterium reacts upon the sugar, acidifies the medium, and breaks the equilibrium in favor of the bacterium. The bacteriophage, not being able to exert its parasitizing action, will be eliminated after a few transfers.

Still another method of separation, the most practical of all, consists in employing the method used by Eliava and Pozerski (described further on) for obtaining cultures of resistant bacteria free from the bacteriophagous ultramicrobe. It is only necessary to make a few passages on agar slants, culturing each time from the extreme upper margin of the agar layer where the medium is somewhat desiccated.

An ultrapure bacterial culture can also be obtained by the use of quinine, since this substance has a higher antiseptic activity for the bacteriophage than for the bacterium.

We have seen that in the case of a bacteriophage but slightly virulent the addition of the filtrate in which it is present to some bouillon does not impair the development of the inoculated bacteria; it is only by spreading cultures on agar that we can detect the presence of the bacteriophage through the plaques which develop there. To increase the virulence of such an inactive strain we have seen that it is necessary to make several successive passages of the bacteriophage along with the bacterium. Between each passage it is essential either to filter the mixed culture through a bougie to separate bacteria and ultramicrobes or to heat the mixture to 60°C. to destroy the bacteria, while leaving the bacteriophage unharmed. What is the basis for this technic? The elimination of bacteria which, because of contact with the bacteriophage, are defending themselves and are acquiring a certain degree of resistance, a resistance which permits them to subsist in spite of the progressive increase in virulence of the bacteriophage.

With each passage, therefore, we force a bacteriophage of increasing virulence to act upon an organism of normal resistance, that is, upon a bacterial suspension lacking resistance at the moment when it is placed in contact with the bacteriophage. Briefly stated, this technic is simply a method of opposing the development of resistant bacteria by natural selection.

THE CHARACTERS OF MIXED CULTURES

Bacteriophage of low virulence

The means whereby the equilibrium obtaining in mixed cultures can be disturbed have been mentioned. It is of interest to allow the struggle to proceed naturally and to note its issue in a medium where each organism is dependent upon its own resources, that is, to permit the natural selection of such bacteria as are most apt in the struggle. To witness this, it is only necessary to make repeated transfers in broth without intermediary filtration or heating. In cases where the bacteriophage is of low virulence *in vitro* the bacterium usually triumphs; its resistance increases little by little, the more vigorous bacilli survive and multiply, and the point is reached where the ultramicrobes no longer find bacteria susceptible to invasion. When this occurs the bacteriophage ceases to multiply and gradually becomes eliminated from the culture, until only a normal culture of bacteria remains.

In some cases the state of equilibrium is more stable and the mixed cultures are able to continue throughout a large number of passages.

Often these mixed cultures show cultural abnormalities and a partial lysis. The medium may become turbid only to become somewhat cleared later and finally to revert to a turbid condition.

Experiment XXIII. Bouillon is inoculated with a mixed culture (*B. dysenteriae*—bacteriophage) taken from an agar slant planted thirteen months previously with a secondary culture. The macroscopic appearance passes through the following stages: after forty-eight hours, uniform turbidity; after five days, almost completely cleared; after thirteen days, uniformly turbid; after nineteen days, slightly cloudy with some sedimentation; after one month, uniformly turbid with sedimentation. This is the final appearance and at this time the bacterium and the bacteriophage coexist in the medium.

Transplants into bouillon continue to give mixed cultures, cloudy, with less marked but definite changes in appearance. These alterations in appearance are separated by intervals of only a few hours.

This same experiment is performed with another strain of anti-dysentery bacteriophage, the inoculation being made from a colony on a secondary culture two months old. After three days there is uniform turbidity. In five days the medium is almost limpid. After eleven days it is very cloudy and after eighteen days it is clear with a slight sediment. All subcultures remain sterile and the medium contains a very active bacteriophage.

In the mixed cultures with changing appearance the struggle between the bacteriophage and the bacterium inclines first in favor of one contending force and then to the advantage of the other. The final issue is at times in favor of the bacteriophage, at times in favor of the bacterium, and the number of transfers necessary to bring about a final issue is extremely variable.

In vitro, the struggle generally ends with the bacterium the victor. In Part II of this text we will see that *in vivo*, with mixed cultures showing fluctuations, the issue of the struggle is determined in some measure by the superior organism (that is, the animal body) in which the contending forces are operating.

Bacteriophage of very high virulence

With a bacteriophage of very high virulence secondary cultures are relatively rare, and when they appear they offer a very characteristic aspect, at least in so far as *B. dysenteriae* is concerned. The medium remains perfectly limpid, the bacterial culture appears agglutinated, multiplying slowly in the bottom of the tube or deposited on the walls. These agglutinated masses may attain a size as large as the head of a pin and they can not be dissociated by shaking. With other bacteria the agglutination is less marked.

Subcultures from these agglutinated mixed cultures give, indefinitely it appears, mixed cultures always presenting the same appearance.[2]

[2] As one of the consequences, very important from the practical point of view, we will see that numerous so-called pure bacterial cultures are to be found in most laboratories which are in reality mixed cultures, contaminated from the time of their origin with a bacteriophage.

Two different strains of anti-dysentery bacteriophage have been preserved for over two and a half years and during that time they have undergone more than 100 successive passages. Nevertheless, during this period, repeated tests have shown in these cultures the constant coexistence of extremely virulent ultramicrobes and of bacteria completely refractory to several very virulent strains of the anti-dysentery bacteriophage.

In these mixed cultures there is a stable equilibrium between the virulence of the one and the resistance of the other, and in such cultures the changes in appearance previously noted will never be observed. They might, indeed, be spoken of as "symbiotic cultures,"[3] for the bacteriophage can not be cultivated in series unless it multiplies and it can not multiply unless it parasitizes bacteria. Moreover, it is only necessary to disturb the equilibrium in favor of the bacterium, by such means as have been mentioned, to cause a rapid disappearance of the ultramicrobes, rendering them henceforth incapable of cultivation.

All of the bacteria present in an agglutinated culture are to be found in the agglutinated clumps, none are free in the medium. Subcultures into bouillon from the clear fluid always remain sterile, no colonies develop when transferred to agar, and microscopic examination fails to reveal any formed elements. All the bacteria there present have assembled in the agglutinate. The clear fluid contains only the extremely virulent ultramicrobes, as may be proved by the inoculation of a bacterial suspension which quickly becomes lysed. On the contrary, as we have seen above, a bouillon subculture made from the agglutinate always results in the growth of a mixed culture.

When a mixed, agglutinated culture is inoculated into a pure culture of the bacteriophage, that is, into a suspension previously inoculated and which has undergone complete lysis, the growth consists of an agglutinated culture, just as though the inoculation had been made into fresh sterile bouillon.

[3] This is possible if we interpret symbiosis in a broad sense, as Noel Bernard has. The definition of symbiosis given by this author applies admirably to mixed cultures: "An intermediary condition at which two antagonistic organisms arrive, with an equilibrium of their forces, tolerating each other in a prolonged common existence."

If some of the agglutinate, even if washed, is introduced into a suspension of *B. dysenteriae* lysis takes place and the suspension becomes perfectly clear within five to six hours. Four or five days later, however, the agglutinated masses begin to appear and gradually increase in size. The ultramicrobes contained in the agglutinate used as inoculum provoke the lysis of the normal bacilli of the suspension, bacilli which are non-resistant, and then later the resistant agglutinated bacilli in their turn reproduce and the result is that which would have been secured had they been inoculated into fresh sterile bouillon.

All stages intermediary between these two extremes may be obtained; cloudy mixed cultures presenting the appearance of a normal bacterial culture where the equilibrium is essentially unstable; cultures in agglutinated form in the presence of a perfectly limpid fluid, representing a state of stable equilibrium. The medium may be more or less cloudy with the bacterial masses more or less compact, sometimes having but little density forming a coagulum. The type of the mixed culture bears a relationship to the virulence of the bacteriophage and to the resistance of the bacterium. Hence, the appearance of the mixed culture may be as variable as is the variability in the properties of the two organisms which are present.

Mixed colonies on agar

Instead of seeding the mixed cultures into broth they may be inoculated on to a solid medium.

Often the agar will remain sterile, indicating that the equilibrium has been disturbed in favor of the bacteriophage. As has been said, this reaction may occur with inoculation into broth, but it is more frequent when agar plantings are made. It appears that the bacteria on agar are more readily attacked than when in a fluid medium. For this there may be several reasons, the principal one doubtless being the proximity of the bacteria. The first phase of the struggle is certainly associated with the phenomenon of chemotaxis. In a fluid medium the bacteria in suspension are separated by considerable spaces, certainly considerable when compared with the diameter of the ultramicrobe. Upon solid media, on the other hand, the bacteria actually touch each other,

and the passage of the parasitic agent from one bacterium to another is readily accomplished since a strong chemotactic force is not required to bring together the two organisms in the struggle. When a culture develops on agar, the appearance of the growth may show considerable variation, determined by the relation between the resistance of the bacterium and the virulence of the bacteriophage. These two factors being inherently variables afford an infinite number of possible combinations, resulting in an infinite variation in the possible appearances on agar. At first sight, one of two principal distinguishing aspects may be present:

1. A smooth layer of culture, *always located in the upper portion* of the agar slant where the medium is less thick (although, it is needless to say, the mixed culture may be distributed over the *entire* surface of the slant). The extent of this covering layer is variable. Sometimes there is only a fringe of bacterial growth at the extreme upper margin of the medium, the remaining portion being sterile. At other times the culture layer covers one-tenth, one-fourth, one-third, one-half, or even three-fourths of the slant, the portion remaining sterile *always* being the lower section of the slant where the agar is of greatest depth. The cause of this is simple. We have seen that the colonies of the bacteriophage appear as circular plaques, apparently sterile, and that they are of greatest size where the agar is deepest. The reason for this has been stated, and the present instance is but an application of this general fact.

Certainly most bacteriologists will recall having seen cultures of this character without having recognized the cause. As a matter of fact, many cultures are to be found among culture collections which are in reality nothing but mixed cultures. In all cases, when one observes abnormal cultures, presenting the characteristics which have been described, one may be sure that such a mixed culture is present, that is, the culture is one which is infected with the bacteriophage.

2. The culture may consist of more or less numerous isolated colonies, even when the medium has been abundantly inoculated.

These isolated colonies, in their turn, may present different appearances, associated always with the degrees of resistance and of virulence of the bacterium and the bacteriophage respec-

tively. Three types of colony may develop, each presenting individual characteristics.

a. The colonies may be those of normal dysentery bacilli.[4] These are encountered especially when working with mixed cultures derived from the inoculation of a suspension with a bacteriophage of low virulence. But even with a very virulent bacteriophage all of the colonies may appear quite normal.

b. Rare colonies, formed only of cocci, and cultivable under this form. They may be grown in bouillon, where an abundant culture of homogeneous turbidity is secured, or on agar, where the colonies appear somewhat different macroscopically from those of a normal bacillus, being more convex and more opaque. Subcultures obtained by the inoculation of these colonies are not mixed cultures; they contain only cocci, no ultramicrobes being present. The coccoid form is maintained during a number of generations and then the bacterium gradually reassumes its normal form.

c. The colonies may be mucoid, refractile, difficult to dissociate, and of very diverse size—from the limit of visibility up to those with a diameter of about one millimeter. These colonies are cultivable on agar and reproduce colonies of the same form. They are mixed colonies, and in them the simultaneous presence of both elements, bacterium and bacteriophage, can always be demonstrated.

Even when abundantly seeded upon agar these colonies never give a smooth layer of growth but always isolated colonies, more or less abundant, and always of variable size. Among the bacteria of the inoculum but few are able to form colonies. There is always a state of unstable equilibrium between the two elements present: the bacterium with its resistance, and the bacteriophage with its virulence. The bacterium forms, or does not form, a colony according to the accidental predominance of one or the other of these factors. This is especially to be observed when agar is seeded with the agglutinated masses, for however abundant may have been the planting only very rare isolated colonies, all of the mucous type, develop.

[4] *B. dysenteriae* Shiga is simply taken as an example; all other bacteria give mixed cultures and mixed colonies showing quite similar appearances.

The cultures secured by the inoculation of the mucous colonies on different media show the following reactions:

In agar stabs: small lenticular colonies about the needle track.

In gelatine: as in agar, the resistant bacteria remain alive and cultivable for at least eleven months. In the case of the Shiga dysentery organisms this represents a viability at least ten times as great as that of the normal bacillus.

In gelatine stabs: large opaque colonies with opaque centers.

On glycerine potato (prepared as for the cultivation of *B. tuberculosis*): very rare colonies on the potato, very abundant growth in the fluid at the bottom of the tube.

In milk: not coagulated in ten days.

In litmus milk: turns to mauve after two months.

On coagulated serum: no growth.

In neutral red: no change in two months, either on agar or in bouillon.

In litmus milk (Petruschky): acid after ten days and remains acid.

When the mucous colonies are suspended and heated to 60°C. they are not cultivable, for then the culture contains only the living very virulent ultramicrobe which is not killed until a temperature of about 75°C. is reached. Reinoculated into bouillon, the refractile, mucous, mixed colonies yield two types of culture, (*a*) mixed cultures showing changes in turbidity, and (*b*) agglutinated cultures, which, as we know, always depend upon the degree of virulence of the bacteriophage and the capacity of resistance of the bacterium, factors which regulate the appearance of the culture.

We have seen that if an agglutinate, taken from a mixed culture in stable equilibrium, is introduced into a suspension, a lysis of the suspension is followed by a growth of the agglutinate. The same thing transpires if an abundant seeding is made on tubes of slant agar having a growth of the Shiga bacillus. First, plaques appear, and then after three or four days a mucous colony develops in the center of each plaque. In both instances the bacteriophage acts upon the normal non-resisting bacteria and dissolves them, then the refractory bacilli multiply as they would have done on a sterile agar or in bouillon.

THE RESISTANT BACTERIUM

From that which has preceded it may be deduced that the acquisition of resistance by a bacterium is reflected in a marked

change in morphology. We have seen that certain colonies on agar are composed of bacteria presenting the coccoid form, other colonies presenting the refractile appearance and a mucous consistency.

Coccoid form

The following experiments are interesting for they allow of the appearance of the coccus form with a later return to a normal morphology—the first in the colony itself at a few days interval, the second in the course of successive passages.

Experiment XXIV. A Petri dish is heavily inoculated from an agar culture of *B. dysenteriae* and is placed in the incubator at 37°C. for about four hours. A drop of the bacteriophage culture is then placed in the centre of the plate. The strain of bacteriophage should be one of average activity, that is, one capable of regularly causing complete lysis of a bacterial suspension but with which secondary cultures usually develop. (With too virulent a strain the area where the drop was placed remains sterile indefinitely.) The plate is returned to the incubator. After eighteen to twenty-four hours a layer of culture composed of normal dysentery bacilli develops, showing in the centre a spot devoid of growth, apparently sterile. After thirty-six to forty-eight hours, the spot becomes covered with extremely fine colonies, which, when examined microscopically are composed of cocci only. These cocci are of different sizes, from 1 to 4 μ in diameter, arranged in irregular forms,—in diplo- and in tetrad groupings. Two days later microscopic examination still shows cocci, but among them are bacillary forms in great number. Subcultures on to agar always give isolated colonies, each colony always reproducing with the same appearance and with the same sequence of forms,—first a coccoid culture, then a mixture of cocci and bacilli. These cultures always contain, moreover, bacteriophagous ultramicrobes.

Eliava and Pozerski have indicated a method for obtaining the coccoid, resistant bacteria free from admixture with the bacteriophage.[5] These bacteria perpetuate themselves under this form for a certain number of generations. Return to the bacillary form occurs gradually and after about fifteen transplants the culture acts as a normal dysentery organism sensitive to the bacteriophage.

[5] The method permits the purification of a mixed culture by the elimination of the bacteriophage. To accomplish this it is only necessary to make transfers on agar with the mixed culture, taking for inoculum in each passage material from the top margin on the agar, as near the edge as possible.

To a suspension of bacteria in bouillon is added 0.01 cc. of a bacteriophage culture and a drop of the material is spread on an agar slant. Frequently the medium remains sterile, as has been shown above. Sometimes a slight fringe of culture is secured, always at the upper margin where the medium is somewhat dried out. Material from this fringe is planted on a tube of sterile agar and after incubation a culture layer studded with plaques is found. A third transfer is made from the upper portion of the tube, and this is continued until an apparently normal culture is secured. That is to say, until the growth develops without plaques. At that time, the culture consists of resistant bacteria, free of bacteriophage, and appearing coccoid in morphology.

In such cultures resistance is maintained during a certain number of passages. Then it gradually decreases and it is observed that this resistance is associated with the coccoid form, for the lengthening of the bacterial elements affords an indication that resistance to the bacteriophage is decreasing.

The coccoid form may be secured in another manner. Certain of the agar tubes seeded with a secondary culture show after a very long time—one or two months, or even more—a colony situated near the top of the agar. This colony increases in size slowly and may attain a diameter greater than one centimeter. It is formed solely of cocci. There can be no doubt but that it consists of modified bacteria since successive subculturings yield normal bacillary forms.

Atypical colonies have been secured with *B. dysenteriae* (Shiga, Flexner, and Hiss), with *B. coli*, *B. typhosus*, and the paratyphoids.

As is seen, the coccoid form is most certainly a resistant form of the bacterium, and the return to bacillary form taking place gradually, affords an index of the decrease in resistance.

This morphologic transformation is accompanied by a profound change in the properties of the bacterium. Coccoid cultures are not agglutinable by a specific serum. This is also true of secondary cultures and mixed cultures in general. Restoration of agglutinability is coincident with loss in resistance and with the return to normal morphology.

In this connection it has been shown that strains of *B. typhosus*, inagglutinable when derived from the body, are at the same time composed of bacilli which are resistant to the anti-typhoid bac-

teriophage, and further, that when, by subculture on agar, they lose their resistance they also become agglutinable. Inagglutinability appears to be a property of the bacteria which resist the bacteriophage.

The vitality of resistant bacteria is much greater than that of normal bacilli. For example, with the Shiga dysentery organism whose vitality is weak (there are but few strains cultivable after one month, none among the numerous strains with which I have worked have remained alive without subculturing for more than two months), all of the colonies on agar of the resistant Shiga substrain are still cultivable after eighteen months.

The virulence of bacteria which are resistant to the bacteriophage is likewise considerably greater than that of normal bacilli.

Whatever may be the nature of the resistant bacteria, and whatever may be their form, there is no doubt but that they can return to the normal form with normal properties. They then behave as the bacteria of the same species which were used to prepare the initial suspension upon which the bacteriophage acted. In a word, there can be no question either of an accidental contamination or that they are visible forms of the bacteriophage.

With a coccoid culture of *B. dysenteriae* Shiga the following biologic reactions have been effected, reactions which indicate that these cocci conserve the general properties of the normal dysentery bacillus:

a. The injection into a rabbit of such cultures causes the death of the animal with paralysis and intestinal lesions identical with those observed in animals killed by the inoculation of typical dysentery organisms.

b. Rabbits immunized with carefully graded doses of such cultures are protected against a surely fatal dose of typical dysentery organisms.

c. The serum of rabbits which have been treated with injections of coccoid cultures contain an amboceptor which will fix complement in the presence of normal bacilli.

Zoogleic form

Microscopic examination of the agglutinate formed in liquid media by bacteria which are endowed with a high resistance, and also of colonies which are mucoid on agar, show that the

bacteria (their morphology will be discussed shortly) are surrounded by a mucous material. They are actual zoogleic colonies.

As has been said above, there can be no doubt as to the nature of these bacteria, since all biologic reactions show that they react as did the bacteria of the same species which were used to prepare the original suspension. Moreover, in every instance, they revert to normal form. It is only necessary to eliminate the bacteriophage, the cause of the transformation.

It is thus very obvious that the resistance of the bacterium to the action of the bacteriophage profoundly modifies its form. Resistance accompanies a transformation into the coccus form, and when the bacterium, having to defend itself against an extremely virulent bacteriophage increases its resistance, there is formed a mucous capsule which certainly functions by hindering the penetration of the ultramicrobes into the bacterial body. Encapsulated bacteria thus enjoy a refractory state.

The transformation is accompanied by modifications in the properties of the bacterium; increase in viability, enhanced virulence, and inagglutinability by specific sera.

The resistance persists only as long as the bacterium must needs resist the action of the bacteriophage. In the absence of ultramicrobes[6] the resistance gradually falls, somewhat more rapidly when it was not very marked. In extreme cases it disappears after about thirty transfers on agar. This represents a very considerable number of generations.

We have seen that it is experimentally possible to render a bacterium resistant to the action of the bacteriophage and we have indicated different methods which enable us to reproduce this phenomenon at will. It is not an artificial phenomenon but is a reproduction of a natural process which takes place within the organism.

Each strain of a species of a pathogenic bacterium, recently isolated from the body of an individual, offers a different resistance to the action of the bacteriophage, and this resistance, as we know, may extend to an absolutely refractory state. These differences of resistance arise, as will be demonstrated later, in hereditary

[6] Suitable means have been indicated for purifying a mixed culture by eliminating the bacteriophage.

factors which originated in the struggle which took place between the ancestors of this bacterium and the bacteriophage within the body of the infected animal. As is the case of the resistance acquired experimentally, this naturally acquired resistance disappears gradually with repeated culture on laboratory media. Thus, different strains of a single species of bacteria tend to become, by a gradual process, uniform, and after a sufficient number of transplantations all are equally sensitive to the action of the bacteriophage.

It is worthy of note that the degree of virulence of a bacterium is strictly in relation with its degree of resistance to the bacteriophage. We will have occasion to demonstrate this frequently when we come to consider the relation between the bacteriophage and the infections due to bacteria.

MICROSCOPIC OBSERVATIONS

In the agglutinated and the zoogleic colonies certain atypical forms are encountered, which are also to be found, although less abundantly, in mixed cultures in general.

Microscopic examination of preparations stained with thionin or by the Giemsa method shows a variety of forms depending somewhat upon the age of the colonies. Up to the second or third day the pleomorphism is considerable. Together with the typical bacillary forms and the cocci there are elongated bacilli, some as long as fifteen micra, with all intermediary lengths; some straight, others curved at all angles. Some are clubbed, yeast-like. Large and small granules are present together with the débris derived from the destruction of the different forms. All stages intermediary between intact forms and amorphous débris are represented. What is the exact significance of these various forms? They are forms of involution or forms assumed by the bacterium in the development of resistance. That is all that it is possible to say with certainty. However, some of these forms give the impression that the organism in question is reproducing by sporogony.

In old colonies but very few of the bacillary and coccoid forms are to be seen.

The subcultures on agar can be multiplied but the appearance is always the same, and no matter which of the colonies is selected the biologic tests mentioned above always show that they are related to the original bacterium. Further, the possibility of reversion to the bacillary form in pure culture when grown on a glucose agar medium demonstrates conclusively that contamination has not taken place.

How may these facts be explained? Early in the consideration of the phenomenon the hypothesis presented itself that in these secondary cultures a visible form of the bacteriophage was present. But experiment has shown that this interpretation was false. A second idea has developed which unfortunately has not been completely studied for lack of time (the subject of the bacteriophage is so far-reaching that it has seemed more essential to utilize the available time on experiments dealing with the function of the bacteriophage rather than with those of the question of morphology) but is presented only that it may be considered by those who are particularly competent to engage in morphologic studies.

The necessity of bacterial intervention for the production of asci by certain fungi[7] is a condition clearly recognized in mycology. This same situation may intervene among bacteria which are resistant to parasitism.[8]

This hypothesis is perhaps the less improbable since, according to Schaudinn, sexuality is a fundamental characteristic of living matter. It is observed with *B. bütschlii* and with *B. sporonema* (in addition to the usual mode of reproduction by transverse

[7] For example, *Ascobolus furfuraceus* (Moliard, 1903), a fungus of the genus Willia (Sartory, 1902), and an aspergillus growing on the banana (Sartory, 1920).

[8] The following suggests that under the influence of the bacteriophage non-spore-forming bacteria may give rise to filtrable forms. I have noted, although rarely, that a filtrate obtained by passing a secondary culture through a Chamberland bougie (L_2 and even L_3) becomes turbid after some days. Each time that this has been noted the turbidity has been due to the growth of a resistant bacterium such as was present in the secondary culture prior to the filtration. The conditions under which this phenomenon occurs have not been ascertained, thus the observation is simply mentioned without emphasis being placed on its interpretation.

division) where there is noted an autogamous reproduction, which implies, at least, a rudimentary sexuality. In autogamy there are necessarily differentiated elements which fuse. According to Schaudinn the loss of sexuality in bacteria is an indication of a degenerative change resulting from the adaptation to a parasitic existence. In accordance with the hypothesis suggested above, there would be a return to sexual reproduction as a means of defense, under the influence of the parasitism to which the organisms have been subjected. Such sexual reproduction would be, then, extremely frequent *in vivo*, as frequent indeed as a struggle between the bacterium and a bacteriophage occurs in the body. In Part II of this monograph it will appear that this struggle is continuous.

THE ACQUISITION OF RESISTANCE

How can this acquisition of immunity by a bacterium be explained? Numerous experiments have shown that if a certain quantity of a *slightly active* culture of a bacteriophage is introduced into a relatively heavy (1000 to 2000 million per cubic centimeter) suspension of bacilli, the ultramicrobes, readily demonstrated at first by the presence of plaques on plantings on agar, disappear from the medium after an interval of time varying from one hour to two or three days, and that they can not later be demonstrated. Subcultures give normal cultures of bacteria. On the other hand, we have seen that with a very virulent bacteriophage the ultramicrobes disappear from the fluid between ten and twenty minutes after introduction into a suspension, but that they reappear in about twenty times as great a number in from one to one and a half hours later—they have multiplied within the interior of the bacteria. In the case of a bacteriophage of low virulence it seems, therefore, that penetration of the bacteria takes place but that multiplication can not be effected. The bacterium resists and the ultramicrobe is actually destroyed *in vivo*. These parasitized bacteria which "recover" acquire by this an immunity. We will see later that they are even capable of producing antilysins.

Another fact has been sometimes observed which shows that certain bacteria are able to become "carriers." As has been said,

heavy suspensions which are inoculated with a filtrate containing a relatively avirulent bacteriophage give after a few hours absolutely normal cultures on agar, free of plaques. If serial transplants are made of these cultures, inoculations made in such a manner as to yield an even layer of growth, it sometimes happens that after a certain number of transplants, two to four, a very definite plaque appears, which is indeed a colony of the bacteriophage. This is evidenced by the fact that successive passages from this plaque yield a very active bacteriophage. From whence could this ultramicrobe have so suddenly come? The ultramicrobe had remained alive within a bacillus, and at a given moment, it overcame the resistance of the latter and multiplied. Its virulence being increased, the young germs were able to parasitize the neighboring bacilli and form a colony. Any other explanation seems impossible, since, immediately after the inoculation of the bacteriophage, seeding upon agar shows plaques characteristic of the presence of virulent bacteriophagous germs, then these germs completely disappear, the bacteria, however, remaining sensitive to the action of a more active bacteriophage, for perfect lysis is secured if the suspension is inoculated with a trace of a very active culture of the bacteriophage, and finally, the active bacteriophage reappears after a series of subcultures on agar in the course of which all the bacillary cultures have been normal. This germ can only be one of those which had disappeared. The fact, demonstrated by experiment, of the penetration of virulent ultramicrobes into the bacteria, warrants us in thinking that this ultramicrobe (but slightly virulent) has been preserved in a latent living state within the interior of the bacterium. At a given moment the resistance of the bacterium is broken down and infection results.

PRODUCTION OF ANTILYSINS BY BACTERIA

By its mere presence the bacteriophage is not able to dissolve the bacterium. In the following chapter we will see that it acts through the secretion of lysins which, however, can be isolated free from the ultramicrobe.

The following experiment shows that the resistance of the bacterium to the action of the bacteriophage, which amounts to

an actual immunity in the true sense of the word, is in great part due to the fact that the bacteria secrete antilysins which neutralize the lysins.

Experiment XXV. A very active strain of bacteriophage, active for *B. dysenteriae,* is diluted in sterile bouillon, 0.05 cc. to 10 cc. of the medium, and 0.05 cc. of this dilution is introduced into a second 10 cc. of medium. In addition, a very concentrated suspension of *B. dysenteriae* is prepared,—a suspension representing a twenty-four-hour slant agar culture in 6 cc. of sterile bouillon. This suspension is inoculated with 0.05 cc. of the second dilution of the bacteriophage culture and incubation at 37°C. for four days follows. At the end of this time it is evident that the suspension is not lysed (because of the too great quantity of bacilli) but when a drop is planted on agar the medium remains sterile. Therefore the bacteriophage has multiplied. This heavy suspension is filtered through a bougie.

To each of three tubes containing 10 cc. of a weak suspension of dysentery bacilli is added—to the first, 0.05 cc. of the filtrate from the heavy suspension, to the second, 0.05 cc. from the first tube, and to the third, 0.05 cc. from the second. After an incubation period of twenty-four hours it is seen that there is no lysis in tube 1, but lysis is complete in tube 2, while the suspension in tube 3 is still turbid although it clears after forty-eight hours.

But one conclusion is possible. Since lysis did not take place in the first tube, in spite of the presence of a large number of virulent ultramicrobes, and since it was produced in the other two which received infinitely less, there must have been in the filtrate some substance which inhibited the lytic action of the bacteriophage. This was not manifested in the last two tubes because of the dilution. It is likewise because of dilution that the inhibiting substance did not manifest its action on agar. It has already been noted, in the course of the earlier experiments, that lysis was more often perfect if a suspension was inoculated with a minimal amount of the bacteriophage than if the inoculation was massive. The cause for this fact is now clear.

This experiment is in all respects identical with that described in a preceding paragraph, except that there it was accomplished by introducing a small number of ultramicrobes of low virulence into a large number of bacilli. Under these circumstances the bacteriophage is overcome and destroyed by the bacterium. In this last experiment, on the contrary, we have substituted for the small number of germs of low virulence, ultramicrobes of high

virulence. These ultramicrobes develop slowly, but at the same time the very numerous bacteria have had time to adapt themselves and to elaborate a defensive mechanism to the lysins secreted into the medium. They have produced an antilysin which paralyzes the action of the bacteriophage. Moreover, it has been shown that these antilysins are specific, for those secreted by the dysentery bacillus are without inhibiting action on the lysis of *B. pestis.*

The bacteria, in general, react like higher organisms. *B. tetani,* for example, secretes a toxin, and an animal which would be killed by a large dose of this toxin reacts to a small dose by the production of an antitoxin which will neutralize the toxin. The bacteriophage secretes a lysin and the bacterium, which could not resist a rapid attack, produces, when placed in favorable conditions, an antilysin which neutralizes the action of the lysin.

We will further see that the organism reacts to the injection of the bacteriophagous lysins, which in effect, are nothing but foreign bodies of the same nature as the toxins,[9] and, indeed, in a manner quite analogous to that of the bacteria, namely, by the production of specific antilysins.

MULTIPLE CULTURES

It remains for us to consider multiple cultures. In the following chapter we will see that the virulence of a strain of bacteriophage is rarely limited to a single bacterial species. What forms do secondary cultures take when a bacteriophage is forced to act upon a suspension comprising different bacterial species? We will confine ourselves to the most simple case,—a bacteriophage reacting upon two species of bacteria. Let us take as an example a strain of bacteriophage very virulent for *B. dysenteriae* Shiga and but slightly virulent for *B. coli,* as evidenced in the following experiment.

Experiment XXVI. Three tubes of bouillon receive respectively 0.01, 0.1, and 1 cc. of a known anti-Shiga bacteriophage. The three tubes are then lightly planted with *B. coli.* Normal cultures develop in the three tubes. Platings on agar give few plaques. Each of the three cultures is

[9] The organism does not defend itself against a toxin because it is toxic but because it acts as a foreign colloid in the body.

re-inoculated into fresh bouillon. Normal *B. coli* cultures develop. Transfers to agar give two plaques for the first tube, none for the other two. The culture yielding the two plaques is again re-inoculated. A normal culture develops. The bacteriophage has been eliminated.

This strain of anti-Shiga bacteriophage possesses, therefore, an extremely feeble virulence for the strain of *B. coli* under test.

Experiment XXVII. To 10 cc. of bouillon is added 1 drop of a concentrated suspension of Shiga bacilli (this should give a slight turbidity equal to about 50,000,000 bacilli per cubic centimeter) and 1 drop of an equally concentrated suspension of *B. coli*. This double suspension is then inoculated with 0.01 cc. of the anti-Shiga bacteriophage used in the above experiment.

After twenty-four hours there is a slight turbidity. A new passage into a double Shiga-colon suspension is made. Perfect lysis takes place after eleven hours.

The lysed suspension is then introduced, in a quantity of 0.04 cc., into a simple suspension of *B. coli*. Lysis is complete in seven hours.

The ultramicrobes have developed at the expense of the Shiga bacilli, and thus being maintained in the medium they have gradually acquired a virulence for *B. coli*.

In the intestinal tract a bacteriophage never finds itself in the presence of but a single bacterial species. And this experiment permits us to comprehend the process of acquisition *in vivo* of virulence by a bacteriophage for a given bacterium.

CHAPTER III

Virulence of the Bacteriophage

Multiple Virulence.
Persistence of Virulence.
Bacterial Species Attacked.

MULTIPLE VIRULENCE

A strain of bacteriophage freshly derived from the body is rarely active against a single bacterial species. Usually it attacks a certain number of species at this time, and possesses for each of these a variable virulence.

It may be objected that this by no means opposes the conception of a plurality of species in the genus Bacteriophage, each species acting against a determined bacterium. This question will be considered in detail in a later chapter. For the time being, however, it will only be stated that all experimental work, particularly the work with the complement fixation reaction, favors the idea of unicity, whatever may be the origin of the bacteriophage.

There is but one bacteriophage, but as isolated from the organism, there is an infinite number of strains, each possessing the capacity to attack diverse bacteria. Such a strain may possess, for example, a very high virulence for *B. dysenteriae* Hiss, an average virulence for *B. coli*, a low virulence for the Shiga dysentery strain, a very weak activity for *B. paratyphosus B*, and none[1] for the other intestinal organisms tested.

Another strain may be very active for *B. coli* and *B. typhosus*, but slightly active for *B. dysenteriae* Hiss, and inactive for the other bacteria tested.

[1] It is evident that when it is stated that a given strain of bacteriophage lacks virulence for such and such a bacterium it must be understood "a virulence such as may be demonstrated by the present technic." Early in my investigations it was possible to detect only those strains possessing a considerable activity; all others were unnoticed. The technic has since been improved, but at the present time it is most certainly not perfect.

It is manifestly impossible to make a complete analysis of a strain of bacteriophage, for to do so would necessitate a determination of its activity against all known, and even against unknown, bacterial species. With a given filtrate prepared from the intestinal contents, we can affirm that a bacteriophage is found there at the moment of testing because of its activity manifested toward a given bacterium. On the contrary, it is not possible to conclude that none is present simply because the tests were negative. Investigating the activity of the intestinal bacteriophage in a filtrate from the feces of a healthy person a negative result has been obtained in testing against the intestinal bacteria toward which it was expected an activity would be evidenced. The investigation was extended to the most varied bacterial types, and finally a strain of bacteriophage was isolated active against an organism of the Salmonella group (bacillus of hog cholera). This strain of bacteriophage was cultivated in series and an active bacteriophage was thus secured.

A given strain of bacteriophage will vary from time to time, either in the body, as can be demonstrated by isolating the bacteriophage each day from the feces of a patient during the course of the disease and during convalescence, or it may vary *in vitro*, as has been shown in the preceding chapter.

All combinations of virulence are possible, both as to quantity and to quality; that is to say, in the extent of the action against varied bacterial species, and in the intensity of the action for each of the bacteria attacked. It can be readily seen, in view of the infinite number of combinations possible, that two strains of bacteriophage identical in all respects can not exist.

PERSISTENCE OF VIRULENCE

The faculty which a strain of bacteriophage possesses to return to parasitism with a bacterium persists throughout a very great number of passages *in vitro* along with a bacterium of another species. For example, in 1916 a bacteriophage was isolated which was extremely active for *B. dysenteriae* Shiga, of but average virulence for *B. coli*, and but slightly active for *B. typhosus* and the paratyphoid organisms. This strain, which has been used in many experiments, was subjected during the years 1916, 1917,

1918, and 1919 to a large number of passages, somewhat more than 1200, always with the dysentery bacillus. Nevertheless, early in 1920 experiment showed that it had an average virulence for *B. coli* and a very low activity for *B. typhosus*.

The action on the typhoid bacillus of a bacteriophage which had received more than a thousand passages with *B. dysenteriae* is evidently weak. This can be demonstrated by spreading on agar, a procedure which permits the formation of characteristic plaques. If we introduce into a tube of bouillon about ten drops of an anti-dysentery bacteriophage and then a small amount of typhoid culture, we secure, after incubation for eighteen to twenty-four hours, a culture of *B. typhosus* which appears normal, but if a drop is seeded upon agar a few plaques are obtained.

The following observation, made by G. Eliava, is of the same nature, but more typical, for there is a crossed reaction on bacteria but remotely related. A strain of bacteriophage isolated from the pus of an abscess (we will see that under certain circumstances the intestinal bacteriophage may pass into the circulation) and very active against *Staphylococcus aureus*, appeared, even after a series of more than 100 passages with the staphylococcus, to be endowed with a degree of activity for the dysentery bacillus.

Experiment XXVIII. Ten cubic centimeters of a Shiga suspension is inoculated with 0.25 cc. of a filtered anti-staphylococcus bacteriophage. When plated immediately on agar a normal culture of *B. dysenteriae* develops. After incubation at 37°C. for twenty-four hours, 0.02 cc. of this suspension plated on agar gives 4 plaques. This virulence for *B. dysenteriae* was increased by successive transfers in association with this organism.

Such experiments allow us to penetrate further into the phenomenon of virulence of the bacteriophage.

We have introduced into the suspensions, either of *B. typhosus* or of *B. dysenteriae*, several hundred millions of ultramicrobes, all virulent for the Shiga bacillus in the first case, for the staphylococcus in the second. Each plaque on the agar represents a colony derived from a virulent ultramicrobe; virulent in one case for the typhoid bacillus, in the other for *B. dysenteriae*. We have also seen that of the several hundred millions of ultramicrobes

only a few were endowed with a latent virulence sufficient to parasitize the new bacterium offered them and to multiply at its expense.

The persistence of latent virulence is the appanage of certain particularly apt individuals. With each passage, at the expense of the new bacterium, these individuals multiply, their virulence becomes enhanced, and finally, after a sufficient number of passages, a complete lysis of the suspension is secured.

With these facts as a basis, an attempt has been made to adapt to the dysentery bacillus a strain of bacteriophage active for the staphylococcus, although the ultramicrobe at first appeared devoid of all action on the dysentery strain. In this attempt, ten drops of an active anti-staphylococcus bacteriophage were inoculated into a double suspension containing in each cubic centimeter ten million staphylococci and ten million *B. dysenteriae*. After twelve passages (each passage being separated by a bougie filtration and twelve drops of the filtrate inoculated into a fresh staphylococcus-dysentery suspension) the bacteriophage very actively attacked the dysentery bacillus. This property developed very abruptly during the eleventh passage. After a series of twenty passages made in conjunction with Shiga alone the bacteriophage did not possess any activity for the staphylococcus, and it has been impossible to cause it to reassume such activity.

Up to the present time it has been impossible to accomplish the inverse experiment, that is, to cause a bacteriophage active for the Shiga bacillus to acquire a virulence for the staphylococcus.

This inequal persistence of latent virulence of the bacteriophage against diverse species of bacteria may be explained. The intestinal bacteriophage maintains itself in the intestinal tract at the expense of the different intestinal bacteria. The bacteriophage is therefore, in reality a normal parasite of the colon-typhoid-dysentery group and an accidental parasite of the other bacteria against which it acquires virulence in the same environment, as a result of conditions at present undetermined. It can be understood then, that a bacteriophage active, when derived from the body, for an organism rather remote from the colon-typhoid-dysentery group, for example, a staphylococcus, retains

for a very long time, probably indefinitely, the capacity to attack a bacterium of the enteric group, and this in spite of very many passages in conjunction with the bacterium accidentally attacked. On the contrary, a bacteriophage possessing, when derived from the body, a virulence for a bacterium accidentally attacked, loses more or less rapidly this virulence if it is maintained *in vitro* with a bacterium normally attacked, that is to say, at the expense of an organism of the colon-typhoid-paratyphoid-dysentery group.

It may be objected that all these facts may be interpreted, not as a persistence of a latent virulence but as a persistence, through the course of successive passages, of several species of the bacteriophage. In other words, there has been a "contamination" of the anti-dysentery bacteriophage by an anti-typhoid bacteriophagous ultramicrobe in the first case cited, and a "contamination" of the anti-staphylococcus bacteriophage by an anti-dysentery bacteriophage in the second.

Both experimentation and mathematical reasoning show that such an interpretation is false.

In the first example cited, it has been shown that the number of ultramicrobes virulent for *B. typhosus* did not vary during the course of the passages. The number of plaques obtained on agar by inoculation of a suspension of typhoid bacilli inoculated with the anti-dysentery bacteriophage is essentially the same, although the bacteriophage has been subjected to fifty, one hundred, five hundred, or a thousand passages at the expense of *B. dysenteriae*. If the ultramicrobes capable of attacking the typhoid bacillus were an "impurity" their number should diminish gradually in the course of the transfers, each passage being a dilution, and they should quickly disappear.

If one calculates the extent of the dilution, after a thousand passages, of the filtrate which served as the original inoculum for the bacteriophage in question, a figure of such magnitude is obtained that a persistence of anti-typhoid bacteriophagous germs, throughout the series of successive dilutions, is mathematically impossible. One can calculate readily up to the thousandth passage (each passage consisting of the inoculation of 0.001 cc. of filtrate into ten cc. of bouillon). The value of the dilution in

the thousandth tube of the series is represented, in cubic kilometers, by the figure 10^{3982}. To appreciate this incommensurable figure, it is sufficient to say that with only the twenty-second passage, one drop of the original filtrate taken from the feces has been diluted in a number of cubic kilometers of liquid expressed by the number 10^{70}. That is to say, by a number of which the logarithm has for a characteristic 70; which would be a cube of liquid of such size that it would require a billion centuries for a ray of light to pass through from edge to edge.[2]

The action is not, therefore, to be explained as a persistence of anti-typhoid bacteriophagous germs through a series of successive cultures.

According to the conception of Maurice Nicolle a bacterium may be considered as a mosaic of properties. Each of these properties: resistance to heat, vitality, virulence for such and such an animal, etc., is susceptible, within a single individual, of continuous variation. Within a bacterial culture, and at any given moment, no two individual bacteria can be found possessing exactly the same properties. This conception, demonstrated by daily experience, applies moreover to all living beings.

Variation, that is, the property of adaptation, is an attribute of life and of life exclusively. Like all living beings, the bacteriophage adapts itself continually, and in any culture the ultramicrobes which compose it do not all possess exactly the same properties. Some are susceptible of rapid adaptation toward a given bacterium, others toward another organism. A bacteriophagous ultramicrobe is a mosaic of properties.

[2] It is evident that the same mathematical reasoning demonstrates that the bacteriophage is itself a living being. If one would wish to explain lysis as due to the presence of a lytic diastase in the intestinal contents (or, what actually amounts to the same thing, the presence of a co-ferment or a catalyzer in the intestinal tract capable of activating a pro-diastase contained in the bacterium), the diastase or the catalyzer or the co-ferment would be quickly eliminated by the dilution. If we suppose possible the persistence of one of these principles, in spite of the dilution which approximates infinity, and its presence at each point in an incommensurable amount in the liquid, we are endowing this principle with the metaphysics of ubiquity. Any conception of transmissible serial bacteriolysis which does not admit as the origin of the phenomenon an autonomous living being, ends in a mathematical absurdity.

When a bacteriophage is virulent, as it comes from the organism, for several bacteria at the same time, as is the usual case, it is apparent that the virulence present for each of these bacteria is subject to variations with time. This is true, however, the virus may be preserved, whether it is kept, sealed in tubes, in the original intestinal contents or whether it is preserved in the form of filtrates prepared from the fecal material.

When kept *in vitro* certain strains of bacteriophage lose their virulence for a bacterium, toward which they were active when derived from the body, much more rapidly than do others.

Experiment XXIX. Typhoid patient Mor. . . . . Examination of the stool was made at the beginning of convalescence. On August 20th, 1918, the stool was treated according to the method described for securing the bacteriophage. The filtrate is distributed in 0.5 cc. amounts in suspensions of the following bacteria:—*B. dysenteriae* Shiga, *B. typhosus*, *B. paratyphosus A*, *B. paratyphosus B*, and *B. coli*. After 24 hours of incubation these suspensions were planted on agar with the following results:

B. dysenteriae Shiga	Sterile
B. typhosus	Sterile
B. paratyphosus A	Numerous plaques
B. paratyphosus B	Numerous plaques
B. coli	Sterile

Specimens of the feces and of the filtrate were preserved in sealed tubes. On January 22nd, 1919, that is, after 5 months, these materials were examined again:

SUSPENSION	RESULT	
	Freshly prepared filtrate	Original filtrate
B. dysenteriae Shiga	Sterile	Sterile
B. typhosus	Normal culture	Normal culture
B. paratyphosus A	Normal culture	Normal culture
B. paratyphosus B	Numerous plaques	Numerous plaques
B. coli	Numerous plaques	Numerous plaques

In this material the virulence of the bacteriophage for *B. dysenteriae* and for *B. paratyphosus B* remained unaltered during the five months, it diminished for *B. coli*, and disappeared entirely for *B. typhosus* and *B. paratyphosus A*.

It should be noted that the result was the same whether the bacteriophage was preserved directly in feces or in the filtrate, that is, in bouillon. Likewise, it is significant that the degree of virulence has no influence on the preservation or the disappearance of the virulence. It was strong for *B. typhosus* and became negative, it was weak for *B. paratyphosus B*, yet this remained intact.

In the absence of passages, simply as an effect of old age, the virulence of the bacteriophage varies then with time, and indeed in a different manner for the diverse bacteria attacked. It becomes attenuated more quickly for some than for others, and for this no general rule can be fixed. We have seen above, with another strain, that after four years and in spite of passages in contact with the dysentery bacillus, the virulence for *B. typhosus* persisted. The last experiment cited is not only interesting then, in that it shows an attenuation of virulence associated with the lapse of time, but also in that it gives evidence that the loss does not occur in equal degrees for all of the bacteria attacked by one and the same bacteriophage.

BACTERIAL SPECIES ATTACKED

Let us now consider the different bacteria for which active strains of the bacteriophage have up to the present been isolated.[3] For each of these only the peculiarities of the reaction as they are

[3] In certain cases the bacteriophage can serve for the identification of bacteria, as the agglutination reaction is used. In order to apply the test a bacteriophage must be employed which has been subjected to numerous passages at the expense of a particular bacterial type so that the accessory virulences may be attenuated as far as possible. For example, all strains of bacteria which are lysed by a strain of bacteriophage that has been cultivated together with Shiga bacilli, are certainly *B. dysenteriae* Shiga. With certain species, *B. pestis* for example, for which the specificity of the bacteriophage appears high, diagnosis by means of the bacteriophage is particularly conclusive. In this last connection it may be said that since the publication of the French edition of this text, a bacteriophage has been encountered active for *B. pestis* and equally active for the bacillus found in pseudotuberculosis of the guinea pig. Thus, it is necessary to recognize the lack of an absolute specificity, limiting somewhat the value of the reaction as applied to the identification of bacterial species.

encountered in dealing with the bacterium in question will be mentioned. As far as general characteristics are concerned, all are similar, that is, what has been recorded in preceding chapters regarding the method of isolation, the mode of action, the variable virulence, the enhancement of virulence by passage, the resistance of the bacteria, and secondary cultures, applies to all strains of bacteriophage and to all species of bacteria attacked. In all of the experiments mentioned up to the present time the dysentery bacillus has been taken as an example, but it has been shown that all such experiments may be repeated with identical results with any strain of bacteriophage active for a definite organism. *B. dysenteriae* presents practical advantages in experimentation. It is easy to isolate a very active bacteriophage for this organism and bacteriologists can readily procure strains and repeat these experiments without conducting a long series of preliminary investigations.

B. dysenteriae Shiga

For this organism it is particularly easy to isolate a very active strain of the bacteriophage. The bacteriophage opposed to this bacillus exists, it may be said to be normally present, in the intestinal tract of numerous animals, the horse and domestic fowls in particular. It is likewise frequent in man and may acquire a high virulence, not only in convalescence from an attack of dysentery, but in recovery from a variety of pathologic conditions. Up to the present time about 200 strains have been isolated, without finding any two of exactly comparable virulence and of equal extent in their action on the related bacteria of the colon-typhoid-dysentery group. Among these 200 strains, one only, and that of a moderate activity when isolated, has failed to act upon any other bacteria of the group.

A strain of bacteriophage active for *B. dysenteriae* Shiga is usually active for *B. coli* and for *B. dysenteriae* Flexner and Hiss. From the point of view of the bacteriophage the Shiga type of dysentery bacilli represents a homogeneous species, a bacteriophage active for one strain being equally active for all others. A bacteriophage very active for one strain of bacilli, at the expense of which it has passed through a number of passages, may

be less active for a freshly isolated race, but after four or five passages in contact with this strain a virulence is acquired equal to that possessed for the first one.

We have seen that the strains of Shiga bacilli which resist the action of the bacteriophage are extremely toxic, possessed of a great vitality, inagglutinable by a specific serum, and actively ferment maltose.[4]

B. dysenteriae Hiss

An anti-Hiss bacteriophage is frequently found in the normal intestine. A bacteriophage showing an activity for any member of the colon-typhoid-dysentery group frequently shows a virulence, more or less pronounced, for the Hiss bacillus.

B. dysenteriae Hiss represents a homogeneous species from the point of view of bacteriophagous activity.

Secondary cultures reinoculated into litmus sugar media do not ferment the sugars in the same way as do normal bacilli. Media containing glucose, maltose, and mannite become acid after ten days; those containing lactose, levulose, saccharose, and also glycerine remain alkaline. After a month the lactose, saccharose and levulose media remain alkaline. Secondary cultures, and also mixed cultures, give the indol reaction but do not react on either neutral red or lead acetate. The resistant bacilli are inagglutinable, have a high viability, and are more virulent for man. In Part II of this monograph we will consider a case of *B. dysenteriae* Hiss septicemia in which the bacillus was resistant to the action of the bacteriophage.

B. dysenteriae Flexner

The anti-Flexner bacteriophage is found in the normal intestine of vertebrates as frequently as are the other strains of bacteriophage.[5]

[4] Pottevin has shown that the normal Shiga bacillus definitely, although somewhat weakly, ferments maltose. Resistant bacilli ferment the sugar much more energetically.

[5] The presence of an active bacteriophage in pathologic conditions is not considered here. This phase will be discussed in Part II of this text.

All strains isolated, active for the Flexner bacillus, have likewise been active for *B. coli*, although with some, activity for other varieties of dysentery bacilli was lacking.

With reference to the bacteriophage, Flexner bacilli constitute a homogeneous species. Resistant bacilli ferment glucose, levulose, maltose, and mannite. They do not ferment lactose, do not blacken lead acetate in an agar medium, and do not react on neutral red. They form indol. They are inagglutinable by a specific serum and possess a high viability.

The atypical character of certain strains of *B. dysenteriae* when freshly isolated from the organism may surely be ascribed to their resistance to the bacteriophage. Elsewhere we will consider a typical case. Furthermore, this observation is of general significance, applicable not to dysentery bacilli alone.

B. dysenteriae "X"

During the course of these investigations a very great number of specimens of feces, derived from patients with intestinal disturbances, have been examined. And in many cases of gastro-enteritis in adults as well as in infants a bacillus having the following characteristics has been isolated:

When inoculated on litmus sugar agar media it fails to ferment any of the sugars tested (lactose, glucose, levulose, saccharose, maltose, mannite, galactose). It causes no change in lactose and maltose Barsiekow medium, but this medium containing glucose and mannite is turned red. It is agglutinated by convalescent serum in titres of 1:100 to 1:500, is not agglutinated by anti-Flexner or anti-Shiga sera. With a serum which agglutinates the Hiss strain to 1:2500 the "X" strain is agglutinated in dilutions of 1:200. It is non-motile, is morphologically like the other dysentery organisms, is Gram-negative, and is toxic for rabbits.

Several strains of bacteriophage active for this bacillus have been isolated. This bacteriophage is constantly present in the intestine in convalescents who have shown *B. dysenteriae* "X" in their stools during the infection. Strains have also been recovered from the intestinal tracts of healthy animals, both man and other animals. The "X" bacillus constitutes a homogeneous species as regards the bacteriophage.

Certain strains of bacteriophage active for *B. dysenteriae* "X" were likewise active for other species of dysentery bacilli, others were virulent for only one or two among them. When maintained for several generations at the expense of *B. dysenteriae* "X" they almost completely lose their activity for other dysentery organisms.

B. coli

An anti-coli bacteriophage is extremely frequent in the feces of normal vertebrates and invertebrates, but only exceptionally is it found possessed of any considerable virulence. On the other hand, in recovery from the most varied pathologic conditions very active strains can be isolated. *B. coli*, particularly when recently isolated, constitutes a heterogeneous species as regards the bacteriophage. In the presence of a bacteriophage possessing a very high virulence for certain coli strains, other races are hardly touched, some are even absolutely refractory. When taken from the body, *B. coli* always shows a degree of resistance. In the intestine it forms with the bacteriophage a mixed culture. On artificial culture media the resistance decreases very slowly with successive transfers.

With a colon organism of maximum resistance, that is, one which is completely refractory, the colonies on agar are large, white, fluent, exactly like those of the bacillus of Friedländer.

B. typhosus

Quite frequently a strain of bacteriophage showing a slight activity for *B. typhosus* can be isolated from the normal intestine. The isolation of a very active strain is exceptional since such are found only in convalescents. A single strain of bacteriophage may show a very great variation in virulence for different races of *B. typhosus*, certain races being entirely resistant to a given bacteriophage although they may be very susceptible to other strains of the bacteriophage. In such a case there is a natural resistance, a true natural immunity, a condition which can be demonstrated not only for the typhoid bacillus but also for *B. coli*, the paratyphoid bacilli, *B. proteus*, etc. It is because of such reactions that these organisms are spoken of as belonging to

species which are not homogeneous as regards the bacteriophage. Typhoid bacilli may acquire, *in vivo* or *in vitro*, a resistance to the action of the bacteriophage, that is, they may possess an *acquired immunity* (this must not be confused with *natural immunity*) and they become inagglutinable as well as possessed of an enhanced virulence for experimental animals. As has been shown for *B. dysenteriae*, repeated culturing on agar progressively lowers the resistance to the bacteriophage, and coincidently restores agglutinability.

B. paratyphosus A

A bacteriophage showing virulence for this bacillus is relatively frequent in the normal intestine. As regards the action of the bacteriophage, *B. paratyphosus A* strains form a more homogeneous species than do the typhoid bacilli. Just as with *B. typhosus*, the paratyphoid A organisms may acquire a resistance to the bacteriophage, may become inagglutinable, and may show an increased virulence.

B. paratyphosus B

A bacteriophage for this organism is very frequent in normal stools. The resistance of *B. paratyphosus B* places this bacillus intermediary between *B. typhosus* and *B. paratyphosus A*. Resistant bacteria are inagglutinable and are of high virulence. Mixed colonies on agar, in which the bacterium has acquired a high resistance for the bacteriophage, present a viscid appearance resembling *B. Friedländer*.

Salmonella (hog cholera)

One strain of bacteriophage active for this organism has been isolated from a normal man. When derived from the body it possessed an average virulence.

B. typhi murium

The anti-paratyphoid B strains of the bacteriophage are sometimes endowed with virulence for *B. typhi murium*. Very active strains have been isolated from the intestinal tracts of white and

gray rats which were rendered experimentally resistant to the disease caused by the ingestion of cultures of the bacillus. The resistant bacilli are very virulent and can be used for the destruction of gray rats, a large proportion of which resist the action of the ordinary virus. It is possible that human infection may be feared because of this increased virulence.

The transitory appearance of such a bacteriophage in the blood of several infected white rats has been demonstrated. Such rats were resistant to infection.

B. proteus

Two strains of bacteriophage very active for this bacillus have been isolated from the stools of two infants having a gastro-enteritis. The virulence of these strains was tested against a dozen strains of *B. proteus* of different origins. Only three of the strains tested were affected by these strains of the bacteriophage, the same three in both cases. The other nine proteus strains were non-susceptible. Included in this last group were two strains of *B. proteus* X_{19}.

The lysate secured through the interaction of a bacteriophage on a proteus suspension, is, immediately after the lysis, extremely toxic for rabbits. Indeed, they are killed within a few hours by the subcutaneous injection of but half of a cubic centimeter. After ten days the lysate loses its toxicity.

B. gallinarum (Klein); B. gallinarum (Moore); B. paragallinarum

Characterization of these bacilli will be reserved for the chapter in which avian typhosis is discussed. With the exception of pathogenicity for man *B. gallinarum* presents all the characteristics of *B. typhosus*, including agglutinability to the titre of the serum with an anti-typhoid serum. There are, as we will see, at least three different species of paragallinarum organisms.

The bacteriophage active for *B. gallinarum* is not effective with all species of paragallinarum, nor is the anti-paragallinarum bacteriophage active for the other races. *B. gallinarum* is a very homogeneous species. The anti-gallinarum bacteriophage is constantly present in the intestinal tracts of fowls which resist

infection. It has been isolated from the blood of three fowls which were recovering from the infection. Outside of epizootic foci it has been found in the intestine of healthy animals.

Bact. diphtheriae

Two strains of bacteriophage active for only atoxic strains of *Bact. diphtheriae* have been isolated from the feces of two horses immunized by the injection of *cultures* of diphtheria bacilli. This observation is only mentioned. Lack of opportunity has prevented further examination of these strains, a study which certainly offers much of interest.

Staphylococcus

A bacteriophage active for the staphylococcus has been isolated under the following circumstances. A guinea pig bit my left index finger and on the next day an inflammation appeared which persisted for three days. On the fourth day there was an accumulation of pus, of which about ten drops were secured. When planted directly upon agar there developed one colony of *Staphylococcus albus* and six of *Staphylococcus aureus*. The remainder of the pus was mixed with twenty cubic centimeters of bouillon and placed in the incubator for 24 hours, then filtered through infusorial earth and a bougie. After five passages at the expense of *Staphylococcus albus* lysis was secured. At first the bacteriophage isolated did not show any activity *in vitro* for *Staphylococcus aureus*. It has been possible to develop a virulence for this organism only after about fifty passages in a mixed *Staphylococcus aureus* and *Staphylococcus albus* suspension, following the technic previously indicated for the enhancement of the latent virulence of an anti-staphylococcus bacteriophage toward *B. dysenteriae* Shiga.

Bacterium of barbone

About thirty strains of bacteriophage for this organism have been isolated, of which twelve were extremely active. Their activity was comparable when tested against different bacterial strains, some derived from Italy, others from Indo-China. We

will return to a consideration of this bacteriophage later, in the chapter dealing with barbone (hemorrhagic septicemia of the buffalo).

When isolated from the body all the strains presented an average or feeble virulence toward the different intestinal bacteria. After about a dozen passages at the expense of the bacterium of barbone these accessory virulences became markedly attenuated.

B. pestis

Twelve strains of bacteriophage active for *B. pestis* have been isolated. Eleven of these were secured from the excreta of rats in the different villages of Indo-China where plague was epidemic. The twelfth was derived from the feces of a patient convalescent from plague. This is the only strain which has been maintained. This strain was also active for the bacillus of pseudotuberculosis of guinea pigs.

Bacillus of flacherie

The bacillus in question was isolated from the bodies of silkworms which had died of a disease presenting the characteristics of flacherie in the breeding establishments in Indo-China. The bacteriophage is frequent in the intestine of the healthy worms among a contaminated stock. The activity of this bacteriophage was the same for each of the three strains of the bacillus tested, isolated from three different breeding places.

B. subtilis

One strain of bacteriophage active for this bacillus was secured in the stools of a patient with dysentery. Having but rarely tested the virulence of the bacteriophage toward *B. subtilis* it is impossible to say if this virulence is frequent or exceptional.

Vibrio cholerae

Among about one hundred cases of cholera studied in Indo-China it was possible to observe but one following recovery. In this last, in spite of daily examination of stools, in but a single specimen taken at the beginning of convalescence has a bacterio-

phage active for the vibrio been found. This gave about fifty plaques when planted on agar. In spite of many attempts it has been impossible to cultivate it by serial transfers. None of the fatal cases yielded a bacteriophage.

The diversity of the bacterial types against which, up to the present time, virulent strains of bacteriophage have been isolated, suggests the idea that the activity of the bacteriophage may be manifested toward any bacterial species whatsoever.

CHAPTER IV

The Bacteriophagous Ultramicrobe

Morphology. Viability. Susceptibility to Different Substances. Unicity of the Bacteriophage. Lysins of the Bacteriophage. Opsonic Power of the Lysins.

MORPHOLOGY

The bacteriophagous ultramicrobe is of extreme tenuity. In a medium containing the bacteriophage the ultramicroscope reveals only some very minute brilliant points. Probably each of these points represents an ultramicrobe, particularly since their abundance, in greater or lesser numbers, corresponds somewhat with the counts made upon agar. Its tenuity is such that a medium containing several thousand million ultramicrobes per cubic centimeter appears perfectly limpid. The ultramicrobe is, however, resident in a definite mass, since each element is deposited on agar in distinct points and this mass must be appreciable since the ultramicrobes spontaneously sediment in the course of time.

Experiment XXX. A culture of an anti-dysentery bacteriophage is filtered through a bougie and allowed to stand without moving in a cupboard for eleven months. At the end of this time, specimens of the culture from the surface and from the bottom of the tube are taken with capillary pipettes.

The count of the superficial layers showed 280,000,000 per cubic centimeter.

The count of the deeper layers showed 2,900,000,000 per cubic centimeter.

The ultramicrobe can be sedimented, although incompletely, by centrifugation at very high speed.

Experiment XXXI. Twenty-five cc. of the bacteriophage (antidysentery) are filtered through a bougie and are centrifuged in a Jouan apparatus for 30 minutes at 12,000 revolutions per minute. Counts show the following:

		per cubic centimeter
Before centrifugation		1,750,000,000
After centrifugation	Surface	50,000,000
	Bottom	3,700,000,000

Dialysis through collodion membranes of various permeabilities gives a rough approximation of the size of the bacteriophagous ultramicrobe. As a test, one part of horse serum was mixed with three parts of a culture of the bacteriophage, and the mixture was subjected to dialysis. Whenever the albumin passed through the filter the bacteriophage passed also, and when the permeability was such that the albumin was held back, so also was the bacteriophage.

The bacteriophage then, passes through when the molecule of serum albumin passes and is retained when the latter is held back. It remains for physicists to more exactly determine its true size, and this determination will be of more interest since the bacteriophage is the only ultramicrobe with which such measurement is actually possible, since it is the only one where the elements can be counted. Thus, it may serve to clear up an important point touching the constitution of organized matter. If one calculates the ultramicrobe as being one one-hundredth of a micron in diameter, it ought to contain about twenty molecules of albumin and five or six atoms of sulfur. Physicists have determined the size of the pores in the most dense collodion membranes as being not greater than two millionths of a micron. But the ultramicrobe of avian plague penetrates such a membrane. Each element can not be greater than one five-thousandth of a micron in diameter, hence it would be composed of one-tenth of a molecule of albumin. On the other hand, an ultramicrobe is indeed a markedly complex organism, capable of adaptation, possessing the faculty of secreting toxins,—the diastases,—having in a word, the characteristics of living matter. This in itself implies a relatively complex organism. We find ourselves, then, cornered by an absurdity, for it is impossible to conceive of a complex organism formed of a single molecule, much less of the tenth part of one. It will be much more simple to admit that it is impossible to understand under what aspect life is present in the ultramicrobe and under what form the matter composing it exists.

It is only since the discovery of the bacteriophage that it has been possible to affirm that each ultramicrobe is a material mass capable of multiplication in the form of like masses. Thanks to it, our ideas regarding viruses have acquired some degree of precision. The study of the bacteriophage by physicists would offer findings of extreme interest, for it is the only virus demonstrated by experiment to exist in particulate form, and with this alone is it actually possible to fix dimensions, thanks to the possibility of recognizing the number of elements present in a liquid.

VITALITY

The bacteriophagous ultramicrobe is extremely resistant toward the majority of destructive agents, a property which it shares, moreover, with other ultramicrobes.

The vitality is very great. Filtrates or cultures containing the bacteriophage are still active after six years when preserved in a sealed tube. However, not all of the germs present in a culture show the same degree of resistance. After preservation for four years a culture which originally contained two thousand million ultramicrobes per cubic centimeter contains only about one hundred millions of living organisms. Such vitality is not exceptional, for certain bacteria, not spore-forming, show a resistance of the same order. For example, cultures of *B. coli* are still cultivable after ten years or so, and here also the different bacilli of a culture do not offer the same resistance, for the number of those which survive becomes smaller and smaller with time.

If a culture of the bacteriophage is allowed to evaporate slowly at room temperature it is found that living germs may be found in the few drops of syrupy fluid remaining in the bottom of the tube. Indeed, certain bacteria act in the same way. On the contrary, living organisms are no longer to be found after twelve months in glucose bouillon cultures, although they may still be alive in lactose bouillon.

In fecal material preserved at room temperature in sealed tubes for thirty-four months (September, 1915, to July, 1918) one may recover the living bacteriophage, as active as at the beginning. This experiment has been performed successfully with four specimens of feces from convalescent cases of dysentery.

In a dry state the bacteriophage is resistant for a long time. A fragment of sterile filter paper is saturated with a drop of a bacteriophage culture (anti-dysentery), dried in the air, and preserved in a sealed tube for six months at room temperature. After this time the piece of paper is introduced into a suspension of *B. dysenteriae* and normal, although delayed, lysis is obtained. The ultramicrobes have, therefore, survived. Another ultramicrobe, that of the tobacco mosaic, has the same property. It remains alive for two years in the dried leaves. It is indeed, unnecessary to search for examples of bacteria as resistant as the ultramicrobes. The cocco-bacillus of locusts is a non-sporulating bacillus, but in the cadavers of locusts dead of the disease which it incites in these insects (cadavers dried over sulfuric acid, pulverized, and preserved in sealed tubes for three years). I have shown that the cocco-bacillus remains alive and virulent, for this powder, seeded into bouillon, gives normal cultures virulent for the locust.

SUSCEPTIBILITY TO DIFFERENT AGENTS[1]

Physical agents: Effect of temperature

At the beginning of my experiments I stated that the temperature of destruction of the bacteriophage is about 65°C. Shortly after this Kabeshima in an early report cited 70 to 75°C., and in a later note 70°C. Very recently Gratia and Jaumain have noted that the lethal temperature showed considerable variability; 61 or 62°C. for the lytic principle acting on the staphylococcus and 65°C. for that acting on the colon bacillus.

For testing this point I had taken as a criterion the ability or inability of material subjected to different temperatures to produce lysis of a bacterial suspension. Moreover, this has been the method adopted by the other investigators who have considered this question. In view of these contradictory results, the effect of temperature has been reconsidered, taking as a criterion, not lysis of a suspension in a fluid medium, but the action of a culture on solid media, a procedure much more delicate.

[1] The experiments dealing with the effects of temperature have been made in collaboration with E. Pozerski.

Experiment XXXII. In the following experiments the culture of bacteriophage under test, previously filtered through a bougie, is taken up in capillary pipettes, sealed at both ends, and completely submerged in a water-bath maintained at the temperatures indicated in each experiment. In each series of experiments 8 tubes with culture are maintained for thirty minutes at temperatures of 60, 62, 64, 66, 68, 70, 72, and 75°C.

Anti-Shiga bacteriophage

Two drops of the culture from tubes maintained at 60, 62, 64, and 66°C., when introduced into suspensions of Shiga bacilli, cause complete lysis in less than fourteen hours. The tests repeated with a second strain of Shiga bacilli give identical results. The bacteriophage heated to 68 and 70°C. causes lysis with one strain of Shiga bacilli but not with the other. When heated to 72 and 75°C. the bacteriophage fails to cause lysis.

One drop of each of these suspensions, which had received the bacteriophage cultures previously maintained at 68, 70, 72 and 75°C., and which had not been submitted to lysis, are planted on slant agar. After incubation, all of the cultures, except the last, which is normal, show plaques characteristic of the presence of the bacteriophage.

Serial passages may be effected, thus permitting the enhancement in virulence of the bacteriophage attenuated by the action of temperature. After two such passages, with the ultramicrobe heated to 68 and 70°C., and after three passages with that which was heated to 72°C., lysis in liquid media is obtained.

Comparable experiments have demonstrated that the bacteriophagous ultramicrobes active for *B. dysenteriae* Flexner, *B. dysenteriae* Hiss, *B. coli*, and *B. paratyphosus B*, act in a quite similar manner. With the bacteriophage active for *B. paratyphosus A* attenuation begins at about 64°C. (at least with the strain tested). With that virulent for *B. typhosus* attenuation is already apparent at about 62°C. In all cases, when heated to 75°C. the bacteriophage is completely inactive, either actually destroyed or attenuated to such an extent that its presence can no longer be detected. In all these instances the bacteriophage shows a recuperative power, the virulence being restored when the temperature to which the virus has been subjected is not higher than 72°C.

Anti-staphylococcus bacteriophage

Attenuation of this bacteriophage is already manifest after heating to 60°C. Subcultures of suspensions which have not been lysed show that it is a simple attenuation, for, even with suspensions inoculated with a bacteriophage previously held at 72°C. for thirty minutes, plaques are obtained characteristic of the presence of an active bacteriophage. Moreover, two passages suffice to restore the original virulence to cultures heated to 62, 64, 66, and 68°C. After heating at 70 and 72°C. the attenuation of virulence does not disappear until after six passages. When heated to 75°C. the bacteriophage is deprived of all activity.

It may be concluded from these experiments that all strains of the bacteriophage react to temperature in the same manner. When heated above 60°C. they are attenuated more or less rapidly according to the bacterial species upon which they operate. All are completely killed, or at least paralyzed, at about 75°C.

Chemical agents

The bacteriophagous ultramicrobe will attack bacteria either in the presence or absence of oxygen, or indeed in an atmosphere of either nitrogen or hydrogen.

Antiseptics

It is interesting to study the action of antiseptics for these substances do not act in the same manner on certain ultramicrobes as on ordinary bacteria. While very sensitive to the action of certain antiseptics, they are very resistant to others.

A culture of anti-dysentery bacteriophage in physiological saline[2] is distributed into four tubes. One serves as a control. The second receives mercuric chlorid to a concentration of 1:200, the third receives copper sulfate to a concentration of 1:100, and to the fourth phenol is added in a concentration of 1:100. After contact with these substances for three days the bacteriophage is living in all the tubes. After four days it is killed in the tubes containing the mercuric chlorid and the copper sulfate. After seven days it is killed by the carbolic acid. It remains alive in the control tube.

It is not killed after contact for a week in a fluid saturated with essence of thyme or of cloves, but its lytic action is not manifested there. The same results are secured in media containing chloroform or sodium fluoride (Bablet).

Eliava and Pozerski have determined with precision the lethal limits, in 24 hours, of concentrations of free H and OH ions. The zone compatible with life lies between pH 2.5 and 8.54, corresponding approximately to an acid 1/160 *N*, and a base 1/260 *N*, whatever may be the acid or alkali tested.

[2] Bouillon is not suitable for this work because of the precipitates which form upon the addition of certain antiseptics. These give rise to entirely false results.

It is difficult to make a direct comparison with the limits of resistance of other micro-organisms, the bacteriophagous ultramicrobe is at present the only one for which such determinations have been made.[3]

The action of glycerine is very interesting. The bacteriophage remains alive for at least two years in a fluid composed of equal parts of glycerine and bouillon or physiological saline. A suspension of bacteria in such a medium, a medium in which the bacteria are unable to reproduce, is lysed as perfectly as in ordinary bouillon, yet in a higher concentration of glycerine the bacteriophage is destroyed. Bablet has in fact shown that when 0.5 cc. of bacteriophage is added to 9.5 cc. of glycerine the ultramicrobe is killed in six days. It may be well to recall that glycerine constitutes the best medium for the conservation of the toxins and diastases. The other known ultramicrobes resist, in general, the action of glycerine.

We have seen that a suspension in a glycerine medium may be lysed by the bacteriophage, and, as always, the lysed culture becomes a culture of the bacteriophage. If we allow such a culture to evaporate slowly at room temperature we will finally have a residue composed of glycerine, all the water being evaporated. Under such conditions, the bacteriophage becomes adapted to its environment and remains alive in the glycerine residue, although it is killed if transferred directly from a bouillon culture to concentrated glycerine. Many instances are known of the adaptation of bacteria to antiseptics; and adaptation is a function of living matter.

[3] I may cite, for example, the following findings which have been reported, not on the zone of life, but on the zone of growth.

G. Dernby (Ann. de l'Inst. Pasteur, 1921, **35**, 277) gives the following figures:

Staphylococcus	pH 4.8 to 8.1
B. subtilis	pH 4.5 to 8.5
B. proteus	pH 4.4 to 8.4
B. coli	pH 4.4 to 7.8

The bacteriophage is, therefore, extremely sensitive to the action of bases and acids, since its fatal limit of alkalinity is the same as the limit for growth of ordinary bacteria; its fatal acid limit is not far distant. It is, therefore, more sensitive than bacteria to concentrations of free H and OH ions. This is further evidence of its living nature.

Eliava and Pozerski have shown that the neutral salts of quinine exert an antiseptic action on the bacteriophage, in three per cent solution killing it in thirty minutes, in one per cent, in a few hours. Emetine hydrochlorate and saponine in the same concentrations are without action. This susceptibility to the antiseptic action of quinine is singular in a germ which is otherwise relatively resistant to antiseptic activities. It is hardly possible to deduce that the bacteriophage is protozoan in nature, for quinine exerts antiseptic properties toward many bacterial species, although on the contrary, it is without action on the diastases and toxins.

When a culture is treated with acetone the albuminoid materials of the bouillon are thrown out of solution, and this precipitate encloses the bacteriophagous virus. The greater portion of the virus is, however, destroyed. The bacteriophage reacts like the spore-forming bacteria, which are found, living, in the precipitate. In this connection a curious thing has been noted. Ordinarily acetone is considered a sterile fluid, but it is not necessarily so, since it has been found that several containers of acetone have been contaminated by *B. subtilis*.

Alcohol gives the same precipitate, but the bacteriophage, contrary to that which happens with the virus of the tobacco mosaic, is killed in less than forty-eight hours in 90 per cent alcohol. The precipitate, as we will see, contains the secretory products of the bacteriophage.

UNICITY OF THE BACTERIOPHAGE

In the preceding chapters detailed experiments have been given which show that whatever the bacteria attacked, the ultramicrobes which attack them belong always to the same species. We will return to other proofs shortly. A single statement, grouping these experiments will be given here.

First. Usually a single strain of bacteriophage will attack several species of bacteria at the same time.

Second. A strain of the bacteriophage, continued through more than a thousand passages *in vitro*, always in conjunction with the same bacterial strain, namely, *B. dysenteriae* Shiga, attacks *B. typhosus* and *B. coli*.

It has likewise been shown that a bacteriophage active for the staphylococcus and maintained through more than one hundred transfers with this staphylococcus still possessed virulence for the dysentery bacillus. And it is also possible to effect, *in vitro*, the adaptation for this Shiga bacillus of a strain of bacteriophage active only for *Staphylococcus aureus*. The staphylococcus and *B. dysenteriae* are bacterial species but remotely related and the crossed reaction constitutes an irrefutable argument in favor of the unicity of the bacteriophage.

Third. An antibacteriophagous serum, the properties of which will shortly be considered, contains an amboceptor specific for the bacteriophage, as is demonstrated in the complement fixation reaction of Bordet-Gengou, and this amboceptor is the same for all species of bacteriophage—the anti-dysentery bacteriophage, the anti-plague bacteriophage from man, the anti-plague bacteriophage from the rat, and the anti-barbone bacteriophage from the buffalo, all fix complement in the presence of serum from a rabbit treated by repeated injections of cultures of the anti-dysentery bacteriophage.[4] For this particular experiment strains of bacteriophage were selected which failed to show a crossed reaction *in vitro* with regard to the different bacteria attacked. The complement fixation reaction is specific with respect to species differentiation.

The proofs of the unicity of the bacteriophage are therefore multiple. There is but a single bacteriophage, common to both man and animals, capable by adaptation of acquiring a virulence toward all bacterial species.

As we have seen in the earlier chapters the bacteriophagous ultramicrobe can not be cultivated in any artificial medium. It is an obligatory parasite, capable of reproduction only within living cells. Moreover, this is the case with all known ultramicrobes. The single one making the exception to this rule, the Asterococcus of pleuropneumonia is hardly longer to be considered as an ultramicrobe, since it has been shown to be perfectly

[4] Since the publication of the French edition of this text Bruynoghe and Maisin have confirmed this fact. They have also shown that fixation of complement is also to be obtained with an anti-staphylococcus bacteriophage under the conditions already mentioned.

visible in stained preparations, as a bacterium of minute size. All the parasitic ultramicrobes are intracellular parasites, for the lesions which they produce are, in all cases, characterized by protoplasmic inclusions or alterations in the nuclei of the cells. The bacteriophagous ultramicrobe differs from the other known ultramicrobes only in its elective action for unicellular organisms. The others act in multicellular organisms.

It would be indeed strange that of all living organisms the bacteria alone should enjoy the privilege of absolute immunity. Such an immunity must have seemed remarkable even before the discovery of the bacteriophage, before ultramicrobes sufficiently small to parasitize them had been recognized. The ultramicrobe is in diameter certainly 2000 times smaller than a bacterium of average size, in volume nearly 2000 million times less. In size, one of these ultramicrobes is, to a bacterium, as the bacterium is to a large fly.

It should be remembered, however, that although up to the present time parasitism of bacteria has not been recognized we have for a long time observed and studied many parasites which incite infectious disease among the protozoa. Several examples will be found cited among the works of Metchnikoff.[5]

It may be well to mention a study of Dangeard[6] entitled "Sur les parasites du noyau et du protoplasma," for the facts disclosed by this investigator offer certain analogies to those presented in the preceding chapters. But there are these differences, namely, the parasite of Dangeard attacks a protozoan, and its dimensions are such that it can be readily observed microscopically and therefore classified.

The observations of Dangeard deal with an Oomycete, *Nucleophaga amoebae* Dangeard, which parasitizes the nucleus of *Amoeba verrucosa* Ehr. The *Amoeba verrucosa* has a large, doubly-contoured, spherical nucleus, and also a nucleolus, likewise spherical, whose diameter is about two-thirds that of the nucleus. The substance of the nucleolus is very dense and stains with great

[5] *Leçons sur la pathologie comparée de l'inflammation*, Paris, 1892, Masson & Cie. *L'immunité dans les maladies infectieuses*, Paris, 1901, Masson & Cie.

[6] *Sur les parasites du noyau et du protoplasma*, Le Botaniste, 1894/95, **4**, 199–248.

intensity with various nuclear staining reagents. Between the nucleolus and the nuclear membrane is a space filled with the nuclear fluid.

The zoospore of *Nucleophaga amoeba* first penetrates the protoplasm of the amoeba but it never develops there; it passes into the nucleus through the membrane which it perforates, most certainly through the aid of a dissolving diastase. Dangeard has demonstrated the portal of entrance of the parasite as a minute circular opening, as though made by a punch, persisting after the entrance of the parasite. After its penetration into the nucleolus the parasite resembles a refractile corpuscle, increasing slowly in size in proportion as the nuclear substance disappears. When this nuclear material has been utilized completely the entire interior of the nucleus is filled and the membrane is distended. At this time the nucleus of the parasite, up to the present time single, actively divides and when sporulation is effected there are about one hundred regularly spaced nuclei. About each of these nuclei a zoospore organizes, and a sporangium is thus formed, containing distinct, rounded corpuscles, which contain nuclei at the time of sporulation.

Frequently a single amoeba is parasitized by two or perhaps several zoospores, and in such cases each develops separately and gives birth to a distinct sporangium. When the sporangium reaches maturity the protoplasm of the amoeba disintegrates, the sporangium ruptures, freeing the young zoospores, and these become distributed throughout the medium, ready to parasitize the healthy amoebae in their neighborhood.

It is evident that I have not made any comparison between *Nucleophaga amoeba* and *Bacteriophagum intestinale*, and that these observations are mentioned simply because there is a certain resemblance between the two phenomena of destruction, that of the amoeba and that of the bacterium.

THE LYSINS OF THE BACTERIOPHAGE[7]

It is obvious that the bacteriophage is unable, merely by its presence, to dissolve a bacterium. This action can only be accomplished through the agency of lytic diastases.

[7] The experiments dealing with the lysins have been performed in collaboration with G. Eliava.

In a culture of bacteriophage the lysins which effect the solution of the bacteria ought to remain in solution when lysis is completed. On the other hand, the ultramicrobe does not resist treatment with alcohol. Therefore, in order to obtain lysin it is only necessary to subject the culture of bacteriophage to the classic procedure for the separation of diastases.

If we mix one volume of bacteriophage culture (anti-dysentery) with nine volumes of 96 per cent alcohol, after contact for 48 hours the precipitate which is formed is well compacted and the supernatant fluid may be decanted. The precipitate, which contains the lysins admixed with all the substances of the medium precipitable by alcohol, is almost completely soluble in saline.[8]

Experiment XXXIII. Precipitate a culture of anti-dysentery bacteriophage with alcohol and dissolve the precipitate in a quantity of 0.8 per cent saline equal to the original volume of the culture. Mix equal parts of this solution and bouillon and add a *B. dysenteriae* suspension sufficient to give a slight turbidity. As a control, prepare a tube containing an equal volume of bacilli suspended in a medium half bouillon and half saline. Place these tubes in an incubator at 37°C.

After twenty-four hours the control is turbid, the bouillon containing the lysin is slightly cloudy. Plantings on agar from the two tubes give normal bacillary growths.

After forty-eight hours the control presents the same appearance and agar inoculation gives a perfect growth. The culture containing the lysin is slightly cloudy and inoculations on agar give only isolated colonies. A count shows that there are 22 times less living bacilli in the last culture than in the control tube.

After three days the appearance is the same as after forty-eight hours.

After four days the bacteria begin to develop a resistance to the action of the lysin, the medium becomes cloudy and inoculations on to agar again give a film of growth.

At no time does one obtain on the agar the plaques characteristic of the presence of the bacteriophage, and the action is not continued in series.

The alcohol precipitate therefore contaias a lytic diastase, free of living ultramicrobes. The dissolving action, although definite, is weak; but on the contrary as we will see later, the lysin manifests itself by an extremely powerful opsonic action.

[8] This manipulation should be carried out aseptically, since filtration of the fluid through a bougie considerably weakens its activity.

OPSONIC POWER OF THE LYSINS

In the following experiments the opsonic power has been determined by the method of Wright and Douglas, making a mixture of one part of the fluid of which the opsonic action is to be measured, one part of a suspension of leucocytes, and one part of a suspension of the bacteria against which the opsonic effect is to be determined. The mixture is aspirated in a capillary pipette which is sealed and placed in the water-bath at 38°C. for fifteen minutes. The contents of the pipette are then spread on a slide, stained, and examined.

As reagents we have taken: Guinea pig leucocytes, a culture of an anti-Shiga bacteriophage, and a suspension of Shiga bacilli.

Experiment XXXIV

1. Control.
   Leucocytes, bacilli, ordinary bouillon
   100 leucocytes phagocytize 36 bacilli
   Opsonic index = 1
2. Leucocytes, bacilli, culture of bacteriophage two years old
   100 leucocytes phagocytize 692 bacilli
   Opsonic index = 19.2
3. The same mixture, except the bacteriophage culture is diluted 1:250
   100 leucocytes phagocytize 156 bacilli
   Opsonic index = 4.3
4. Leucocytes, bacilli, culture of bacteriophage six days old
   100 leucocytes phagocytize 1510 bacilli
   Opsonic index = 41.9
5. The same mixture, except the bacteriophage culture is diluted 1:250
   100 leucocytes phagocytize 146 bacilli
   Opsonic index = 4.1
6. The same mixture, except the bacteriophage culture is heated at 60°C. for 30 minutes
   100 leucocytes phagocytize 728 bacilli
   Opsonic index = 20.2
7. The same mixture, except the bacteriophage culture is heated and diluted to 1:250
   100 leucocytes phagocytize 101 bacilli
   Opsonic index = 2.7

In the mixtures 2, 4, and 6, the indices recorded represent a minimum. Many leucocytes contain such a number of phagocytized bacilli that counting is impossible. Since in the above counts only those cells

which did not contain masses of bacteria have been included, the actual index is therefore somewhat higher.[9]

The opsonic action of a culture of the bacteriophage manifests itself with such rapidity that it is improbable that the opsonic power can be exercised directly by the ultramicrobes. We have seen, in fact, that the bacteria are parasitized only after an appreciable lapse of time,—ten to twenty minutes.

Experiment XXXV. Leucocyte suspension, Shiga suspension, and anti-Shiga bacteriophage culture are mixed in equal parts. After various periods of incubation drops of the mixture are examined showing:

TIME INTERVAL	NUMBER OF BACILLI IN 100 LEUCOCYTES	INDEX
Immediately after mixing	197	5.4
After 2½ minutes	362	10.0
After 5 minutes	372	10.3
After 7½ minutes	440	12.2
After 10 minutes	824	23.0

After ten minutes some of the leucocytes are so completely filled with bacilli that counting is impossible. The figure given is a minimum based only on leucocytes in which masses of bacteria were not present to interfere with enumeration.

The opsonic power must be exercised, not by the ultramicrobes, but by the lysin contained in the culture, as the following experiment proves.

Experiment XXXVI. Two milligrams of the alcohol precipitate (of which we have spoken above), still moist, are dissolved in 10 cc. of 0.8 per cent saline. A mixture is made of equal parts of this solution, suspension of Shiga bacilli, and leucocytic suspension. After 15 minutes at 38°C. microscopic examination of stained preparations shows that 100 leucocytes have taken up 536 bacilli (as certain leucocytes contain masses rendering a count impossible the figure is a minimum). The index is 14.9.

The opsonic power of cultures of the bacteriophage is, therefore, due to the lysin secreted by the ultramicrobes, to the lysin which remains in the culture once the bacteria have been dis-

[9] It will be recognized that the opsonic indices obtained with sera are far below these secured with cultures of the bacteriophage. With the former an index of 2 is exceptional.

solved. It is interesting to note its action on bacteria resistant to the action of the bacteriophage.

Experiment XXXVII. Mix equal parts of a suspension of Shiga bacilli resistant to the action of the bacteriophage, an anti-Shiga bacteriophage culture, two years old, and a suspension of leucocytes. After fifteen minutes, 100 leucocytes have ingested 8 bacteria. The index is thus 0.22, or 90 times less than with normal bacilli (experiment XXXIV, 2).

Prepare a similar mixture, but with a bacteriophage culture six days old. Here, 100 leucocytes have phagocytized 13 bacilli. The index is 0.38, or 108 times less than with normal bacilli (experiment XXXIV, 4).

Another mixture is made, using the lysin solution, 100 leucocytes have phagocytized 19 bacilli. The index is 0.53, or, 28 times less than with normal bacilli (experiment XXXVI).

From this it is clear that bacteria which resist the bacteriophage also resist phagocytosis.

The same experiment has been performed with a strain of the antibarbone bacteriophage and the bacterium of barbone. The results are comparable.

Experiment XXXVIII (*A*) 1. Control. Mixture of equal parts of leucocyte suspension, bouillon, and suspension of the bacterium of barbone.

After fifteen minutes at 38°C. there are no bacteria in 100 leucocytes.

2. Mixture of equal parts of leucocyte suspension, the suspension of the bacterium of barbone, and an anti-barbone bacteriophage culture, 8 months old.

After fifteen minutes 100 leucocytes have ingested 109 bacteria.

3. The same mixture, except that the bacteriophage culture is diluted 1:250.

One hundred leucocytes have phagocytized 52 bacteria.

4. Mixture of one-third leucocyte suspension, one-third bacterial suspension, and one-third solution of the alcohol precipitate of a recent culture of the anti-barbone bacteriophage (2 mgm. of precipitate in 10 cc. of saline).

One hundred leucocytes have phagocytized 239 bacteria.

(*B*) A mixture is made of equal parts of leucocyte suspension, culture of the bacterium of barbone, and a fresh (four days old) culture of anti-barbone bacteriophage. During incubation at 38°C. drops taken for examination show:

Immediately, in 100 leucocytes there are 30 bacteria.

After two and one-half minutes, in 100 leucocytes there are 139 bacteria.

After five minutes, in 100 leucocytes there are 201 bacteria.

After seven and one-half minutes, in 100 leucocytes there are 271 bacteria.

After ten minutes, in 100 leucocytes there are 269 bacteria.

In the control mixture made with bouillon no bacteria were phagocytized.

Here it is impossible to calculate the opsonic indices, since no phagocytosis occurred in the control mixture. The indices are infinity.

The bacterium of barbone which resists the action of the bacteriophage is also resistant to phagocytosis.

Experiment XXXIX. Prepare a mixture of one-third leucocytic suspension, one-third of the same culture of anti-barbone bacteriophage as that used in the preceding experiment, and one-third of a suspension of the bacterium of barbone resistant to lysis. After fifteen minutes at 37°C. 100 leucocytes have ingested 3 bacteria, that is to say, 90 times less than with normal bacteria.

The strain of anti-dysentery bacteriophage employed in the experiments previously described manifests a definite, although feeble, lytic action against *B. typhosus.* The following experiments show that it also exerts a definite opsonic action on this bacillus.

Experiment XL. 1. Mix equal parts of bouillon, leucocyte suspension, and *B. typhosus* suspension.

After fifteen minutes at 38°C. 100 leucocytes have phagocytized 68 bacilli. The opsonic index is 1.

2. Mix equal parts of anti-Shiga bacteriophage culture, leucocyte suspension, and typhoid suspension.

After fifteen minutes 100 leucocytes have ingested 203 bacilli. Opsonic index = 3.

3. Mix equal parts of leucocyte suspension, typhoid suspension and lysin solution (the same one as that employed in the experiments with the dysentery bacillus).

After fifteen minutes 100 leucocytes have ingested 109 bacilli. Opsonic index = 1.6.

The lysin possesses a property which is indeed peculiar. When used in the complement fixation reaction as an antibody it functions as an amboceptor. The experiment cited below is taken from among many others which gave identical results.

Experiment XLI. Antigen: This is prepared according to the method of Maurice Nicolle. One loopful of an agar culture of *B. dysenteriae* Shiga is suspended in 4 cc. of saline. This suspension, heated at 100°C. for five minutes, then cooled, serves as antigen.

Antibody: An alcohol precipitate of a culture of anti-Shiga bacteriophage taken into solution in a quantity of saline equal to the original volume of the culture acts as antibody.

The complement is fresh guinea pig serum, titrated.
The hemolytic system is the usual anti-sheep system.

TUBE	ANTIGEN	ANTIBODY	COMPLEMENT	SALINE		HEMOLYTIC SYSTEM	RESULT
	cc.	cc.	cc.	cc.	Incubation in the water-bath for one hour at 37°C	cc.	
1	0.5	0.2	0.2	1.6		1	+ +
2	0.5	0.4	0.2	1.4		1	+ +
3	0.5	0.5	0.2	1.3		1	+ + +
4	0.5	0.6	0.2	1.2		1	+ + +
5	0.5	—	0.2	1.8		1	Complete hemolysis
6	—	0.6	0.2	1.7		1	Complete hemolysis, rapid
7	—	—	0.2	2.3		1	Complete hemolysis
8	—	—	—	2.5		1	+ + + +

By itself, the lysin does not fix complement, on the contrary, as shown by tube 6, it rather activates hemolysis. There is, therefore, an antibody associated with the lysin which fixes the complement.

It is difficult to reach a conclusion regarding this curious experiment. Later investigations will show that there is a relation between the amboceptor of Bordet and the lysin of the bacteriophage, since it acts as two different principles, acting in an identical manner, in so far as complement fixation is concerned.

All of these experiments show that the bacteriophagous ultramicrobe secretes a principle, precipitable by alcohol, resisting a temperature of 58°C., and persisting for several months in the cultures of the bacteriophage. This principle, aside from its solvent action, exercises a powerful opsonic action upon the bacteria for which the ultramicrobe from which it is derived possesses a virulence. The opsonic activity appears proportional to the virulence of the bacteriophage for the bacterium under consideration. Bacteria which have acquired a resistance to the bacteriophage are equally resistant to phagocytosis. In addition, from another viewpoint, we will see that they possess an increased virulence.

CHAPTER V

The Bacteriophagous Antiserum[1]

Complexity of the Antibodies. Antibodies to the Bacteria. Antibodies to the Bacterial Toxins. Antibodies to the Bacteriophagous Ultramicrobes. Antibodies to the Lysins. Incidental Conditions Resulting from the Existence of the Bacteriophage.

COMPLEXITY OF THE ANTIBODIES

The phenomena here involved are exceedingly complex. It is known that when the body is injected with a bacterial culture it responds with the production of diverse principles which are grouped under the name "antibodies." Some of these act upon the bacterial bodies: the agglutinins, amboceptors, opsonins; others, the antitoxins and antiferments, neutralize the secretory products of the bacteria formed in the culture fluid injected. When a mixture of two bacterial cultures is injected, the body responds with a duplicate series of antibodies. This takes place when a culture of the bacteriophage is injected.

A culture of the bacteriophage is composed, as we know, of a culture or a suspension of a bacterium lysed by the action of the bacteriophage directed against and endowed with virulence for this bacterium. The bacteriophagous germs inoculated have multiplied at the expense of the bacterial bodies found there and when lysis is terminated the bacterial substance is dissolved in the medium. A culture of the bacteriophage is, then, a complex medium which contains:

a. The substance of the bacterial bodies in a dissolved state.

b. The bacterial toxins (exo- or endotoxins).

c. The bacteriophagous ultramicrobes which have developed at the expense of the bacteria.

[1] The experiments performed on the bacteriophagous antiserum have been made in collaboration with G. Eliava.

d. The products resulting from the activity of the bacteriophage, which we have grouped under the name of "lysins," and which remain in the medium after the lytic process is completed.

Does the bacteriophage attack the bacterium by means of a single diastase or through a combination of diastases? At this time it is impossible to say, and indeed, it would not materially affect the question with which we are concerned.

There is still another category of substances present in the culture. We have seen that the bacteria do not remain passive to the action of the bacteriophage, and that this defense is accompanied by the production of an anti-diastase—an anti-lysin—which is likewise to be found in the medium. This should, then, stimulate the formation of anti-anti-lysins. These have not been investigated, and it is only sugested that they may possibly be present in the serum of immunized animals.

As an example of an antibacteriophagous serum we will take the antibacteriophage-Shiga serum. This is particularly interesting because of the potent endotoxin of the dysentery bacillus.

A rabbit is injected with four doses of a culture of the anti-dysentery bacteriophage[2] that is to say, of a lysed culture of *B. dysenteriae* Shiga, amounting to two, four, six, and eight cubic centimeters, with an interval of six days between each injection. The rabbit is bled fifteen days after the last injection. Theoretically, the serum of this rabbit ought to contain the following antibodies:

a. Antibodies to the bacteria: Amboceptor and agglutinin.

b. Antibody to the bacterial toxin: Antitoxin.

c. Antibodies to the bacteriophagous ultramicrobe: Amboceptor and agglutinin.

d. Antibody to the lytic diastase of the bacteriophage: Anti-lysin.

Let us see if the antibodies present in such a serum actually correspond with those which theoretically should exist.

[2] As we will see in regard to barbone of the buffalo, the serum of an animal which has received a single and minimal injection of a bacteriophage culture does not present the antibacteriophagous property, or, at least, if it exists, it is not detectable.

ANTIBODIES TO THE BACTERIA

The dysentery bacilli are agglutinated by the antibacteriophagous serum, and this serum contains also an amboceptor which permits the fixation of complement. The presence of such antibodies is inevitable and is obtained by the injection of any material containing the dysentery bacilli, living or dead, intact or dissolved. The presence of such antibodies is without especial significance.

ANTIBODIES TO THE BACTERIAL TOXIN

In the present case these antibodies should neutralize the dysentery endotoxin. The serum of an animal prepared by the injection of dysentery bacilli, living or dead, contains an antitoxin.[3] On the other hand, a culture of *B. dysenteriae* lysed by the bacteriophage contains a toxin, for if experimental animals are injected with such a culture a short time after lysis the animals die as though they had received a lethal dose of the toxin of Nicolle. The serum of an animal treated with such cultures ought to contain an antitoxin. This can be verified.

Experiment XLII. A mouse receives by subcutaneous injection a lethal dose of the dysentery toxin prepared by the method of Nicolle, and at the same time 0.5 cc. of the bacteriophage-Shiga antiserum. A second mouse receives the same amount of toxin and 0.5 cc. of an anti-dysentery serum. A third mouse receives a lethal dose of the toxin only. The first mouse dies in about thirty hours after the injection, the second lives, the third dies four days after the injection.

The bacteriophage-Shiga antiserum is therefore not antitoxic; indeed, on the contrary, it is definitely sensitizing. Let us consider this singular phenomenon further.

[3] The antidysenteric serum furnished by the Pasteur Institute is derived from horses treated by injections of dysentery toxin secured according to the method of Rowland, as modified by Maurice Nicolle. The bacterial bodies are ground with anhydrous sodium sulfate, the powder obtained is dried in the air, and dissolved in water at the time of injection. The turbid fluid thus obtained is centrifuged, and the clear supernatant portion is used for the injection. The serum neutralizes the endotoxin, as animal experimentation shows.

Experiment XLIII. Four mice receive subcutaneously a dose of toxin equal to one-tenth of the lethal dose. The first is held as a control. Two others receive 0.2 cc. of the bacteriophage-Shiga antiserum, the last 0.1 cc. of this serum. The first remains perfectly well indefinitely. The two which received the 0.2 cc. dose of serum die after 40 hours, and the last one after fifty-four hours.

This experiment proves that the bacteriophage-Shiga antiserum sensitizes the animal to the action of the toxin. It should be stated here that whatever the number of lethal doses of the toxin of Nicolle injected into a mouse, death never occurs before the fourth day. Here, when the antibacteriophage serum is added to the toxin, even to a dose below the minimal lethal dose, death takes place within forty-eight hours.

Instead of toxin, let us take living dysentery bacilli and see the effect of the antiserum on injections of this nature.

Experiment XLIV. Four mice receive subcutaneously a dose of dysentery bacilli equal to one-fifth the lethal dose. The first mouse is held as a control. The second receives, subcutaneously, 0.2 cc. of the antibacteriophage-Shiga serum, the last two 0.1 cc. of this serum. The control animal lives, showing nothing abnormal. Those which at the same time received the serum die in seven to nine days after the injection, after showing during the last twenty-four hours a paralysis of the posterior extremities. In general, mice do not show this symptom after the injection of *B. dysenteriae*; only the rabbit shows this particular symptom.

The result is clear-cut; in all cases the antibacteriophage-Shiga serum is sensitizing. This is, incidentally, the first example of an anti-immunizing serum.

It is possible that the antibacteriophage-Shiga serum actually contains an antitoxin but that this is masked by the presence of a powerful "sensibilisine." This is the more plausible, for we shall see in the chapter dealing with immunization, that rabbits which have received but a single minute injection of a culture of the antidysentery bacteriophage are effectively vaccinated against the effects of the toxin. The sensibilisine develops in the animal only after the second injection. This condition is not peculiar to the case of dysentery; we find it again when we consider immunization against barbone.

This phenomenon of sensitization invites much further investigation, which will permit, without doubt, an extension of our

knowledge of the nature of antitoxic immunity. One thing already appears certain, namely, that the bacteriophage must play some rôle in the manifestation of this immunity, since the antibacteriophagous serum sensitizes to the toxins. It is probable that this sensitization of the animal must be associated with the inhibiting power with which the antibacteriophage serum is endowed, an action which we will shortly consider.

ANTIBODIES TO THE BACTERIOPHAGOUS ULTRAMICROBES

Agglutinins

It has been impossible to demonstrate definitely the presence of agglutinins, although experimentation indicates that their presence is probable. In all cases, if present, they are weakly active.

Experiment XLV. When a culture of the bacteriophage is centrifuged for 15 minutes at 3000 revolutions per minute no trace of sediment appears; it requires a speed of at least 12,000 revolutions to obtain an appreciable amount. A culture of the bacteriophage is mixed with one-tenth of its volume of antibacteriophage serum and centrifuged for ten minutes at 3000 revolutions. Counts of the ultramicrobes in the sediment and in the supernatant fluid (after energetic shaking of the latter for five minutes) shows that there are about three times as many germs in the sediment as in the supernatant fluid.

Is this agglutination? It is possible, but not certain. The experiment is not sufficiently clear-cut to permit an affirmative answer. The formation of agglutinins in the serum of treated animals is subject to great variation, dependent upon the bacterial species injected. With *Vibrio cholerae* and with *B. typhosus* for example, potent agglutinins are secured; with certain coliform bacilli and with *B. Friedländer* they are usually so weak that their action is almost inappreciable. We know nothing regarding the formation of agglutinins in the case of the pathogenic ultramicrobes.

Amboceptors

The test for an amboceptor specific for the anti-dysenteric bacteriophage, detectable by the complement fixation reaction

of Bordet and Gengou, can not be made. In effect, the culture of the anti-dysentery bacteriophage is only a suspension of ultramicrobes in a liquid containing the dissolved substance of the dysentery bacilli, so that the antibacteriophage-Shiga serum contains two amboceptors, one specific for the dissolved substance, the other for the ultramicrobe, and it is impossible to separate the two actions. A fixation of complement would always be obtained without the possibility of knowing with which of the two antigens it had been effected. However, the question can be solved in another manner; a manner which is very conclusive.

We have considered in the preceding chapters the experiments which demonstrate the unicity of the bacteriophage. If the hypothesis based on these experiments is true the amboceptor present in the antibacteriophage-Shiga serum ought to fix complement with all ultramicrobes and the detection of an amboceptor ought to be possible, for working with a culture of bacteriophage other than the antidysenteric there would be nothing to interfere with the reaction. A culture of antiplague bacteriophage, for example, is a suspension of ultramicrobes in a fluid containing the substance of *B. pestis*. The only possible common element in a culture of antidysentery bacteriophage and in a culture of antiplague bacteriophage is the bacteriophagous ultramicrobe. And the single "anti" element contained in an antibacteriophage-Shiga serum capable of exercising an action toward such a plague culture can only be an amboceptor for the single element common to all cultures of the bacteriophage; the bacteriophagous ultramicrobes themselves.

The complement fixation reaction has been conducted utilizing as antibody the antibacteriophagous-Shiga serum; as antigens, cultures of bacteriophage for dysentery, plague of human origin, an anti-plague strain from rats, and an anti-barbone strain from the buffalo.

Experiment XLVI. Fixation of complement.

Antigen: culture of anti-Shiga bacteriophage containing 2,000,000,000 ultramicrobes per cubic centimeter.

Antibody: antibacteriophage-Shiga serum.

With the antigen in an amount of 1 cc. and antibody in quantities of 0.2 and 0.1 cc. fixation is positive.

Moreover, the antigen by itself fixed complement.

Experiment XLVII. Antigen: culture of anti-Shiga bacteriophage containing 100,000,000 ultramicrobes per cubic centimeter.

Antibody: antibacteriophage-Shiga serum.

Positive fixation was secured with mixtures containing the antigen in 1 cc. amounts and the serum in 0.2 and 0.1 cc. quantities.

The antigen by itself did not fix complement.

Experiment XLVIII. Antigen: *B. dysenteriae* Shiga.

Antibody: antibacteriophage-Shiga serum.

Fixation of complement occurred.

Experiment XLIX. Antigen: culture of the anti-Shiga bacteriophage.

Antibody: an antidysentery serum.

Complement is fixed.

Experiment L. Antigen: culture of the anti-Shiga bacteriophage.

Antibody: antibacteriophage-Shiga serum.

The antigen, antibody, and complement are incubated together for 1 hour at 37°C., and then the hemolytic system is added.

The following protocol shows the results.

TUBE	ANTIGEN	ANTIBODY	COMPLEMENT 1:20	SALINE	HEMOLYTIC SYSTEM	RESULT
	cc.	cc.	cc.	cc.	cc.	
1	0.75	0.2	0.2	0.35	1	+ + + +
2	0.50	0.2	0.2	0.60	1	+ + + + (optimum)
3	0.25	0.2	0.2	0.85	1	+ + +
4	0.75	0.1	0.2	0.45	1	+ + + +
5	0.75	0.3	0.2	0.25	1	+ + + +
6	0.75	—	0.2	0.55	1	Complete hemolysis
7	0.75	0.2	0.3	0.25	1	+ + + (excess complement)
8	0.75	0.2	0.4	0.15	1	+ + (excess complement)
9	—	0.3	0.2	1.00	1	Complete hemolysis

Experiment LI. Antibody: antibacteriophage-Shiga serum.

Antigens: Four different antigens are employed, as follows:

I. Anti-Shiga bacteriophage, 1,500,000,000 ultramicrobes per cubic centimeter.

II. Anti-barbone bacteriophage, 250,000,000 ultramicrobes per cubic centimeter.

III. Anti-plague bacteriophage, a strain derived from the rat, 450,000,000 ultramicrobes per cubic centimeter.

IV. Anti-plague bacteriophage, a strain derived from a case of plague in man, 700,000,000 ultramicrobes per cubic centimeter.

The results secured with these four different antigens are shown in the following table.

ANTIGEN	ANTIBODY	NORMAL RABBIT SERUM	COMPLEMENT 1:20	SALINE	HEMOLYTIC SYSTEM	ANTIGENS			
						I	II	III	IV
cc.	cc.	cc.	cc.	cc.	cc.				
1	0.2	–	0.2	1.1	1	++++	+++	++++	++++
1	0.1	–	0.2	1.2	1	++++	++	+++	++++
–	0.2	–	0.2	2.1	1	C H	C H	C H	C H
1	–	–	0.2	1.3	1	C H	C H	C H	C H
–	–	–	0.2	2.3	1	C H	C H	C H	C H
1	–	0.5	0.2	1.3	1	+	+	+	+

++++ = Complete inhibition C H = Complete hemolysis

Experiment LII. The antibody and the antigens are the same as those used in the preceding experiment. The following table presents the results.

ANTIGEN	ANTIBODY	NORMAL RABBIT SERUM	COMPLEMENT 1:20	SALINE	HEMOLYTIC SYSTEM	ANTIGENS			
						I	II	III	IV
cc.	cc.	cc.	cc.	cc.	cc.				
1	0.05	–	0.2	1.25	1	++++	+++	++++	++++
1	0.025	–	0.2	1.30	1	++++	++	++	++++
1	0.005	–	0.2	1.30	1	+++	C H	C H	C H
1	–	0.05	0.2	1.25	1	C H	C H	C H	C H
1	–	0.025	0.2	1.30	1	C H	C H	C H	C H
1	–	0.005	0.2	1.30	1	C H	C H	C H	C H
–	0.05	–	0.2	2.25	1	Complete hemolysis			
–	–	0.05	0.2	2.25	1	Complete hemolysis			
1	–	–	0.2	1.30	1	C H	C H	C H	C H
–	–	–	0.2	2.30	1	Complete hemolysis			
–	–	–	–	2.50	1	No hemolysis			

It will be noted (experiment LI) that the antigen slightly fixed complement with normal rabbit serum. This is not strange since the bacteriophage is a normal inhabitant of the intestine.

The antibacteriophage serum contains, therefore, an amboceptor specific for the bacteriophage, whatever the strain may be, whatever the species of bacteria attacked, and whatever the animal species from which it is derived. There is but one bacteriophage.

ANTIBODIES TO THE LYSINS[4]

We have already seen that it is possible to obtain lysins without admixture with viable bacteriophagous ultramicrobes by precipitating a culture of the bacteriophage with alcohol.

Since growth of the bacteriophage takes place within the interior of the bacteria the ultramicrobe can effect its penetration only by corroding the wall of the bacterium in order to make way for its passage, and it is evident that it can do this only by means of a lysin. If the antibacteriophage serum contains an antilysin it is evident that the penetration will be retarded or even prevented by the presence in the medium of the neutralizing serum. And this delay or prevention, according to the amount and potency of the serum, will be associated with a delay or prevention of the lysis, since the ultramicrobes will be unable to penetrate the bacterial cells. The antibacteriophage serum will assure, in a word, the protection of the bacteria, without actually exercising any action on the vitality of the virus itself. This is, in fact, what is actually observed.

The antibacteriophage serum used in these experiments possessed a considerable inhibiting power; 0.00001 cc. added to 10 cc. of a suspension of dysentery bacilli—this is one-millionth of the final volume—markedly retarded lysis. With 0.001 cc. lysis was prevented. Obviously, this fact might be interpreted as a destruction of the bacteriophage pure and simple. But this would be indeed strange in view of the fact that a serum has never destroyed a bacterium *in vitro*, even in the presence of complement. I know that this affirmation is contrary to universal opinion touching the bacteriolytic action of antisera, but in Part II of this work I will show the foundation for it by an experiment which permits of no doubt.

In so far as the action of an antibacteriophage serum upon lysis is concerned, the following experiment shows that the bacteriophage is not destroyed; its action is only inhibited.

[4] To repeat what is meant by lysin: The aggregate of the secretions of the bacteriophage, without prejudging that they operate as a diastase only, or as a collection of diastases, as is more probable. It has been shown in an earlier chapter that the bacteria, like higher organisms, react to the lysins by the production of antilysins.

Experiment LIII. Prepare a mixture of equal parts of antibacteriophage-Shiga serum and culture of anti-Shiga bacteriophage. Allow them to remain in contact for five days. They are placed under conditions such that if the serum exerted a destructive action on the ultramicrobes its effect could not fail of manifestation. After these five days of contact, three tubes, each containing 10 cc. of sterile bouillon, are seeded with a drop of a bouillon culture of Shiga bacilli. To the first of these tubes add a drop of the mixture of antiserum-bacteriophage; to the second add a drop from this first tube after it has been shaken; and to the third add a drop from the second. We have then a series of three tubes, planted with *B. dysenteriae* Shiga, containing decreasing concentrations of the serum-bacteriophage mixture. After twenty-four hours at 37°C. normal cultures of Shiga are obtained in the three tubes, and plantings on agar likewise give normal cultures. Up to this point it looks as though the lytic principle has been destroyed.

Continue the experiment. Replace the three tubes in the incubator and twenty-four hours later it is seen that lysis has commenced in the first tube of the series. Agar inoculation from this tube remains sterile. The last two, on the contrary, still contain a normal culture of *B. dysenteriae*. Return the tubes again to the incubator. After twenty-four hours, that is, three days after the beginning of the experiment, lysis takes place in the last two tubes. All subcultures on agar remain sterile.

The bacteriophage is therefore not destroyed by the antibacteriophage serum; its power is simply inhibited for a time.

The experiment further shows that the action is truly inhibitive, acting upon the entire number of ultramicrobes. In other words, the delayed lysis is not due to the revival of certain ultramicrobes particularly resistant, since lysis is produced even in the last tube which has received, as a result of successive dilution, an infinitesimal quantity of the germs.

The presence of an antilysin in the antibacteriophage serum allows us to obtain further information regarding the nature of the virulence of the ultramicrobe.

Experiment LIV. To a suspension of the bacterium of barbone add a drop of a bacteriophage culture active against this organism and then 10 drops of an antibacteriophage-Shiga serum, that is, a quantity of serum completely inhibitive of lysis in a suspension of Shiga bacilli. Prepare also a control without the serum. In both tubes lysis proceeds normally and in a parallel fashion. Here, then, the serum has exerted no inhibitory action.

The inhibitive effect is exercised only against the strain of bacteriophage which has been used to inject the animal in the preparation of the antiserum.[5] On the other hand we know that strains of the bacteriophage differ one from the other only in the virulence which they have acquired, by adaptation, for this or that bacterium. It follows that the lysin secreted by the bacteriophage is different for the different bacteria attacked, since the antilysin neutralized only the lysin of the strain which has served in the treatment of the animal furnishing the serum. Each bacterial species requires for its lysis, then, the production of a specific lysin.

Virulence for a given bacterium is, therefore, in the last analysis, the power possessed by the bacteriophage to secrete a lysin specific for this bacterium.

The bacterial species are divided into groups. In each group the species which compose it present certain common characteristics. For example, we have the colon group, the typhoid group (*B. typhosus* and the paratyphoid bacilli), the dysentery group (the Shiga, Flexner, and Hiss types, etc.). These three groups are indeed closely related one to another. On the other hand, we see the Pasteurella group (bacteria of the diverse hemorrhagic septicemias, chicken cholera, barbone, etc.), the staphylococcus group, and so on. Each bacterial species requires that it be attacked through the secretion of a specific lysin and the difference between these specific lysins will be slight in passing from one bacterial species to another in the same group or in a neighboring group. The bacteriophage adapts itself rapidly. A single strain is in fact generally active for all the bacteria of the group, or for the organisms of the most closely related ones. On the contrary, adaptation is difficult of acquisition in passing from a bacterium of one group to an organism of a remotely related group.

Furthermore, the bacteriophage normally parasitizes the intestinal bacteria which constitute its habitual culture medium.

[5] It is evident that if the antibacteriophage-Shiga serum is tested against closely related bacterial types, forms for which the bacteriophage would have a certain activity, an inhibitive effect more or less pronounced will be noted.

It retains for a long time its hereditary faculty of attacking the organisms of the colon-typhoid-dysentery group, even if it is cultivated for many generations at the expense of another bacterial species.

INCIDENTAL CONDITIONS RESULTING FROM THE EXISTENCE OF THE BACTERIOPHAGE

I wish to note certain incidental consequences which spring from the facts which we have considered. Although accessory, these consequences are of some practical significance and it may be well to mention them.

Because of the ubiquity of the bacteriophage and its constant presence in all living beings, and because of the resistance which the bacteria are able to oppose to its action, and which gives birth to the phenomenon of mixed cultures, it is henceforth necessary to verify the purity of bacterial cultures from the point of view of possible contamination not only by another bacterial species, but also by an ultramicrobe.

We will see, for example, that in *B. coli* pyelonephritis, the pathogenic agent is always a colon bacillus which is resistant to the bacteriophage. If one plants the urine from a case of pyelonephritis on agar for the purpose of isolating the etiological agent one is always liable to find mixed colonies of the colon bacillus and the bacteriophage. Subculture from these mixed colonies will give mixed cultures, indefinitely cultivable in this form. Such cultures are usually considered pure, for they contain no other organism visible under the microscope. They are, however, contaminated by the bacteriophagous ultramicrobe; they are not "ultrapure," and investigations undertaken with such mixed colon-bacteriophage cultures may furnish peculiar results, especially if they are used in immunological experimentation. It is not to be assumed that I have mentioned an exceptional case, far from it, as the two following examples demonstrate.

In two different instances, both accidental findings, I have demonstrated that cultures of the colon bacillus isolated originally from cases of cystitis in the hospital, were in reality mixed cultures. In both instances it was easy to isolate from these cultures a very active bacteriophage.

Here is another instance of the same order. Recently investigating a bacteriophage active for the streptococcus of gourme of horses, Doctor Forgeot sent me three different strains of *Streptococcus equi*, taken from the culture collection of the Central Veterinary Laboratory. Of these three strains, only one was pure. The two others were in reality mixed cultures of the streptococcus and the bacteriophage.

These two examples suffice to give an idea of the great number of mixed cultures which must actually exist among stock cultures. Not only are the intestinal bacteria subject to contamination by the bacteriophage, but bacteria in general, for we will see that the bacteriophage does not remain restricted to the intestinal tract, but passes into the circulation and exercises its action in the different organs.

It is therefore quite essential before undertaking any experimental work to ascertain if the bacterial culture involved is not in reality a mixed culture, composed of a resistant bacterium and a bacteriophage. One must be sure that the culture is not only pure, but ultrapure.

The mutations noted by different authors may most certainly be attributed to the frequency of these mixed cultures. And the confirmation of these reports has been lacking for the very simple reason that the verification has been attempted, not with strains contaminated by the bacteriophage, but with cultures really pure. The experimental results have thus very naturally differed. In this regard I might say that it is indeed singular, in view of the unique character of certain conceptions concerning the bacteriophage, that not a single author has yet traced to the fact that bacterial cultures are frequently contaminated by the bacteriophage, the conclusion that the bacteriophage takes origin spontaneously in these cultures. In reality, as we see it, in connection with the genesis of these cultures, each time that there is a contamination by the bacteriophage, it is in the form of a mixed culture. The bacteriophage exists in the culture from the beginning, that is, from the time of isolation.

In the experiments touching immunity, the contradictory results may likewise well reside in the presence of a bacteriophage in the cultures employed in the experiments. We will see in the

second part of this work, the rôle which the bacteriophage plays in immunity, and it is evident that the experimental results in immunological investigation will be entirely different if the bacterial strain employed is, or is not, contaminated by the bacteriophage; whether it is a resistant strain or a normal strain.

In a word, the idea of the existence of the bacteriophage imposes the obligation of always verifying the bacterial cultures with a view to determining that they are, not simply pure, but ultrapure; and this under penalty of obtaining entirely false experimental results as a result of the possible presence of the bacteriophage.

CHAPTER VI

The Nature of the Bacteriophage

Nature of the Bacteriophage. The Number of Possible Hypotheses. Experimental Proofs of the Living Nature of the Bacteriophage. Refutation of the Hypothesis of Kabeshima. Refutation of the Hypothesis of Bordet and Ciuca. Refutation of the Hypothesis of Bail. Refutation of the Hypothesis of Salimbeni. Conclusions.

THE NATURE OF THE BACTERIOPHAGE

All of the facts which have been recognized up to the present time and which have been recorded in the preceding chapters have been confirmed by all authors who have investigated the question. The phenomena themselves have never been the subject of controversy, and because of their definiteness, it might be said because of their violence, and because of the facility with which they can be reproduced, they can not be controverted.

The discovery of the bacteriophage was associated with a study of a disease of locusts, in which I for the first time noted in the intestine of the insects which resisted the infection a principle antagonistic to the action of the pathogenic cocco-bacillus; a principle which could be demonstrated by its effects but which of itself could not be isolated. With this suggestive observation as a basis, I systematically sought for a comparable principle in the intestinal contents of patients with enteric infections. Finally, during the year 1915, in studying an epidemic of dysentery which prevailed in a squadron of cavalry stationed in the neighborhood of Paris, I noted the phenomenon of plaques in the cultures on agar tubes. Shortly after, from the stools of a patient under treatment in the Pasteur Hospital I was able to isolate by filtration the antagonistic principle. The study was continued during the rare moments which my duties as Chief of the Laboratory Service for the Preparation of Vaccines permitted. (More than twenty million doses of vaccines were furnished by the Service to the Allied Armies during the war.) I tried particularly to

determine the nature of this principle, and it was only after I had considered all possible *a priori* hypotheses, multiplying the control experiments which had demonstrated experimentally with certainty that the principle could be only an autonomous living being,—a filtrable microbe parasitizing the bacteria,—that I resolved to publish, in 1917, the first communication announcing the discovery of an ultramicrobe parasitizing the dysentery bacillus. In this report I gave the principal characteristics of the virus and indicated the rôle played by it in the course of the disease. From 1917 to the end of 1919, I continued these investigations alone, extending them to other diseases, and it was only in December 1919 that Kabeshima, working in my laboratory with strains which I had furnished him, published results which confirmed mine. Since that time, investigations on the bacteriophage have multiplied, and rare indeed are the laboratories which are not interested in the question.

It may seem strange, at first sight, that I should have been able to work alone on this question for such a long time; a circumstance which has permitted me to establish the facts in their entirety, and the relation between them; to demonstrate their importance from the point of view of immunity, and to accomplish this before any other communication appeared. In this I have been favored by circumstances and even by the strangeness of the facts themselves, which at first excited, not merely astonishment, but incredulity, even among my most friendly colleagues, who were not loath to consider me a visionary. This time has passed. The facts are recognized to be correct. The most recent work appears to affirm the curative properties of cultures of the bacteriophage. The only point at issue is my conception of the nature of the active principle.

I may be permitted to make a few remarks upon the subject of this discussion. All authors, without exception, who have formulated an hypothesis regarding the nature of the bacteriophage have adopted a method of reasoning that is somewhat peculiar. None of them have taken the trouble to review the experiments that I had accumulated in favor of the living nature of the bacteriophage during the years that I had alone been occupied with this question; experiments moreover, which in no instance are open

to question. Each of them has taken simply a particular fact, suited to support his thesis, and has neglected entirely the great group of experimental facts which render this hypothesis inadmissible, forgetting that that which accords with experiment is, for a theory, the sole and indispensable criterion of its truth.

But the strangeness of their procedure is not restricted to the interpretation of their experimental findings, but extends to the experiments themselves. These experiments correctly performed react against their hypotheses. I have called attention to these errors and Kabeshima seems to be converted by the evidence, for he has published nothing for two years. As for Bordet, he does not maintain that his fundamental experiment, called that of leucocytic exudates, may be repeated. He has recognized that the specificity of the bacteriophage, a condition *sine qua non* for his hypothesis, is contrary to fact, but he nevertheless continues to support a hypothesis thenceforth without foundation.

THE POSSIBLE HYPOTHESES

The number of possible hypotheses is limited, and after a consideration of these fundamental hypotheses it is only necessary to select that which accords with the observed experimental facts which have been contradicted by no one. These hypotheses were carefully reviewed prior to all publication in an attempt to determine the nature of the principle which I had discovered.

Three fundamental hypotheses can be formulated and any other view must be a modification or a combination of one or more of these three. Discussion of these three must necessarily dispose of any subsidiary hypotheses that may be advanced.

What, then, are these three fundamental hypotheses which comprise all that can possibly be formulated?

First hypothesis

The bacteriophage is derived from the superior organism in its reaction to the bacterial invasion by the production of a principle which provokes the destruction of the bacterium.

This first hypothesis admits of two solutions.

1. The principle in question is a substance of diastatic nature. The single fact of the serial action of the principle is sufficient to

reject this explanation, for such a substance would be rapidly eliminated in the successive dilutions during repeated transfers. It is thus useless to discuss this further. Up to the present time no one has recommended this.

2. The active principle derived from the organism reacting against the infection is particulate, an organic being, capable of developing afterward outside of the organism at the expense of the bacteria.

This hypothesis does not constitute a scientific heresy, for it is not contradicted by any experimental fact. In the case of any hypothesis, however improbable it may appear in view of the actual state of biologic science, if it can not be experimentally demonstrated false and if it harmonizes with the demonstrated facts, it can not be rejected *a priori*. Moreover, Carrel has shown that it is possible to cultivate tissues outside of the organism; and in addition, Altmann has proposed a theory according to which the zymogenic granulations can be nothing but bioplasts, independent elements, having their individual existence and capable of reproduction by division in a cellular medium. The bacteriophage may be a bioplast, derived from the superior organism, and capable of multiplication at the expense of the bacteria.

However this may be, since this particle, this "organite," comports itself from the time when it is taken from the organism as an autonomous being capable of assimilation and reproduction, and since it acts as a being corresponding to the definition of a microbe, it must be a minute being endowed with life. We will revert to this idea in the case of the third hypothesis.

Second hypothesis

The bacteriophage may be derived from the lysed bacterium itself.

The two subsidiary hypotheses given above may again be formulated: 1. The bacteria secrete a diastase with autolytic functions. As we will see, this is in effect the conception of Kabeshima. Bordet takes over this hypothesis with an added complication, since he explains the origin of the principle in terms of the first hypothesis, that is, a substance derived from the organism, and explains the continuity in series by means of the

second hypothesis, that is, to a substance derived from the bacterium itself.

This hypothesis fails before the following experimental facts:

a. The bacteriophage exists in the form of particles which multiply.

b. The bacteriophage does not exercise a specific action upon any single species of bacteria, but at one and the same time, the same bacteriophage may be active against several species.

c. All strains of the bacteriophage, whether they be active against the staphylococcus, against the dysentery bacillus, against *B. pestis*, or against any other organism, belong to the same species, as demonstrated by the complement fixation reaction.

2. The bacteriophage is derived from the bacterium, but is particulate, an "organite" capable of life, conducting itself thenceforth like an autonomous ultramicrobe. This is the hypothesis of Bail.

While an interpretation which comprehends an "organite" derived from the superior parasitized organism does not necessarily imply specificity of the bacteriophage, any hypothesis involving an "organite" derived from the bacterium which is subjected to lysis does imply a strict specificity. The last view, therefore, is inadmissible, since experiment proves that a single strain of the bacteriophage may be active toward several species of bacteria at once, and that all strains of the bacteriophage belong to the same species.

All possible hypotheses, save that of the ultramicrobe, which we will shortly examine, can only be some combination of the two preceding ones. All, then, will be subject to the same objections; none will be admissible for it will be contradicted by experimental facts.

Third hypothesis

The bacteriophage is an autonomous organism, an ultramicrobe parasitizing the bacteria. This hypothesis is the only one which accords with all the recorded experimental facts, and it is for this logical reason that I have attached myself to it. For it, I have acquired more and more convincing evidence which I have not been able to disprove, for the numerous new facts that I

have discovered harmonize with this hypothesis, with this hypothesis only, and none contradict it.

Moreover, I should repeat that I have not formed any hypothesis as to the species to which the ultramicrobe belongs. I have called it *Bacteriophagum intestinale*, a name simply denoting its characteristic property and the place where I first found it. Is it a protozoan? Is it a bacterium? Does it belong to a kingdom which is neither vegetable nor animal? Does it arise even in another organism, a possibility which has been suggested in examining the first hypothesis? These questions can be ignored. It is an ultramicrobe, a filtrable being endowed with the functions of assimilation and of reproduction, functions which characterize the living nature of beings and which pertain to them alone. That is all that experimentation is actually able to demonstrate.

To try to penetrate further into its identity would be nothing but purely speculative reasoning.

EXPERIMENTAL PROOFS OF THE LIVING NATURE OF THE BACTERIOPHAGE

This section will, without doubt, be judged unnecessary by the reader who has followed the experiments recorded in the preceding chapters. However, it may be well to group these proofs and to comment on certain experiments which can leave no doubt, even in the minds of the most skeptical and uninformed.

1. The bacteriophage proliferates, since serial cultures can be continued indefinitely. In the action of the diastases there is always a certain proportionality. The action is the more energetic when the amount employed is large. With the bacteriophage this is not true. The activity is due to the quality of the principle, not to its quantity, and this is, indeed, a property of vital activity. A diastase acts in proportion to its quantity, a bacterium in proportion to its virulence.

2. The bacteriophage presents properties analogous to those of other known living beings. Its resistance to agents of destruction, although great, is, however, less than that of many organisms of which the living nature is unquestionable and uncontroverted.

I ought in this connection to dwell upon the action of temperature, since some authors have suggested that the temperature of destruction of the bacteriophage was too high to allow a consideration of them as living beings. It is thus necessary to recall some of the elementary facts which readers of this work certainly ought not to ignore. The lethal temperature, as we have seen, is about 75°C. Without mentioning the living organisms from thermic sources, of which the temperature reaches up to 93°C., it is known that one may readily isolate from sewage bacteria which develop normally at a temperature of 75°C. Moreover, Duclaux has shown that the young cells of *Tyrothrix tenuis* do not die until a temperature of about 100°C. has been reached. A lethal temperature of 75°C., far from being exceptional, must be recognized as well below that resisted by a large number of unicellular organisms.

In so far as the action of antiseptics is concerned, the bacteriophage takes a position intermediate between the bacteria in their vegetative form and the spores derived from these bacteria. More resistant than the first, they are more sensitive than the second. Compared from this point of view with other known ultramicrobes, they are definitely more susceptible than some. The virus of the tobacco mosaic, for example, will resist for several months a concentration of alcohol which will kill the bacteriophage in a short time. The virus of rabies, and that of vaccinia, remain alive in concentrations of glycerine that destroy the bacteriophage.

It is to be noted that the bacteriophage presents the characteristic of being particularly sensitive to certain reagents which have absolutely no effect upon the diastases; quinine for example. As for glycerine, which destroys the bacteriophage, it constitutes the medium of choice for the indefinite preservation of bacterial toxins and the most sensitive diastases.

3. With sufficiently active strains of the bacteriophage a complete and permanent lysis is secured; all of the bacteria contained in a suspension are definitely destroyed. Moreover, serial passages of the bacteriophage are possible in bacterial suspensions made in fluids which do not permit the development of these bacteria;—physiological salt solution, or bouillon with forty per

cent glycerine, for example. This proves again that the survival of a certain number of bacteria does not constitute a factor in the serial activity, for, as this factor would fail the series could not be continued.

4. On agar the bacteriophage gives colonies at the expense of the bacteria, and this permits counting the active elements. A soluble ferment, diastase or toxin, can not concentrate its action in definite points. It may be objected that the lytic diastase is provided by the bacteria themselves and that each plaque on agar represents the area where is to be found, after the inoculation, a bacterium particularly able to furnish the diastase under the influence of a force "X."

The following experiments demonstrate that this objection is not valid.

Experiment LV. (*A*). Take 10 tubes. Into the first place 10 cc. of a suspension of *B. dysenteriae* Shiga containing 100,000,000 bacilli per cc., into the second place 10 cc. of a suspension containing 200,000,000 bacilli per cc., into the third place 10 cc. of a 300,000,000 suspension, and so on, increasing by 100,000,000 the concentration of the suspensions introduced into each tube of the series. The tenth tube, then, will have a suspension containing 1,000,000,000 bacilli per cc. Each of these tubes is then inoculated with an equal quantity of a very dilute culture of the bacteriophage filtered through a bougie, about 0.000005 cc. We have then a series of 10 tubes containing a more and more concentrated suspension of *B. dysenteriae* Shiga in the ratio of 1:2:3:4:5:6:7:8:9:10, and an equal amount of bacteriophage culture. The tubes are carefully shaken, and 0.02 cc. from each tube is planted upon agar slants. After incubation at 37°C., each of the 10 tubes of agar shows a culture of *B. dysenteriae* spotted with plaques, and the number of these plaques is practically the same for all the tubes.

(*B*) Take 10 tubes, each containing 10 cc. of a suspension containing 100,000,000 *B. dysenteriae* per cc., that is, a like suspension in all 10 tubes. To the first add 1/100,000 cc. of filtered bacteriophage culture, to the second 1/200,000 cc., to the third 1/300,000 cc., 1/400,000 cc. to the fourth, and so on, each tube receiving a smaller and smaller amount of bacteriophage culture, so that the tenth tube will contain only 1/1,000,000 cc. Thus, we have a series of 10 tubes, all containing an equal number of dysentery bacilli and an amount of bacteriophage culture varying according to the ratio 10:9:8:7:6:5:4:3:2:1. Shake the tubes thoroughly and plant 0.02 cc. from each of the 10 suspensions on to agar slants. After incubation at 37°C. each of the agar tubes will show a covering growth of *B. dysenteriae* studded with plaques, but the number of plaques in the tubes varies with the proportion of bacteriophage culture which has been added. Prac-

tically, their number varies from tube 1, which had received the largest amount of bacteriophage culture, to tube 10, which had received the smallest, in the proportion of 10:9:8:7:6:5:4:3:2:1.

These two experiments, which complement each other, demonstrate unquestionably, that the active principle is contained solely in the filtered culture of the bacteriophage; and that this active principle is composed of material elements, capable of forming colonies on agar at the expense of the surrounding bacteria in such a way that their enumeration is possible. These material elements are capable of multiplication as is shown by the formation of colonies and by action in series. It can thus only be a living organism. This experiment by itself is sufficient to demonstrate that the bacteriophage is a "formed ferment," which in reality implies the existence of an ultramicrobe parasitic of the bacteria.

5. Eliava and Pozerski have shown that toward concentrations of free H and OH ions the range fatal for the bacteriophage is more limited than for the bacteria. The diastases and toxins react in a wholly different fashion.

6. Dumas, confirmed by Beckerich and Hauduroy, have isolated the bacteriophage from the soil and from the filtered water of streams. I have myself isolated it from sea-water. There is nothing strange in this, since it is an ultramicrobe derived from stools. This fact can not, on the contrary, be reconciled with a hypothesis of a ferment, whether the ferment be of leucocytic or other origin.

7. Diastases in solution are absorbed by the precipitates which form upon the addition of alcohol. This takes place with cultures of the bacteriophage, but the elements which precipitate are not the ultramicrobes themselves. The ultramicrobes are destroyed by the alcohol, and the principle which is precipitated will not reproduce the action in series. This possibility of extracting from a culture an active principle which can only be a secretory product of the bacteriophage shows indeed that the latter can be nothing other than a living being.

8. I have shown that the bacteriophage is capable of adaptation to the harmful action of glycerine. Adaptation is the appanage of living beings exclusively.

9. It is impossible to isolate two strains of the bacteriophage which are identical in the intensity of their action or in the scope of their activity. With a single strain the intensity of the action can be varied experimentally. Variation is an exclusive characteristic of life.

10. The lytic action is always exercised by one and the same element as is demonstrated in the complement fixation reaction. This element adapts itself to parasitism toward such and such a bacterium, and the possibility of such adaptation necessarily implies the living nature of the element which exercises it.

11. Bruynoghe and Maisin have shown that the bacteriophage is phagocytized and destroyed by the leucocytes. This fact shows that the bacteriophage is foreign to the organism, and it alone demonstrates that it can not be of leucocytic origin.

The nature of the bacteriophage is not open to question; its origin alone may be disputed. Is it an entirely autonomous being, a "species", botanically or zoologically? Is it a "bioplast" capable of indefinite reproduction at the expense of living bacteria, conducting itself as an autonomous being of which it has all the properties?

It is still impossible to decide this; experiment alone will determine. However this may be, the two hypotheses, although they may differ as to the *origin* of the bacteriophage, agree as to its *nature.* The most probable interpretation is that the ultramicrobe is autonomous, a botanic or zoologic "species."

REFUTATION OF THE HYPOTHESIS OF KABESHIMA

Without doubt readers will wish to know the diverse hypotheses proposed by those who have opposed the living nature of the bacteriophage, and I will discuss them in their chronological order, although this may not be their documentary standing.

Apart from the confirmation of the experiments which I alone, or with my collaborators, have effected, these discussions represent about all that has been done on the question of the bacteriophage. By indicating these and commenting on them, this work becomes completed and is a comprehensive statement of the present knowledge regarding the bacteriophage.

Kabeshima, in 1920, starting on the one hand from considerations without significance (as, for example, the thermal death point of the bacteriophage, which he found to be too high to be applied to living beings), and on the other hand, from inexact experimental results (he announced, for example, that the bacteriophage resisted the action of alcohol; that it was active in the presence of sodium fluoride, etc.), formulated the following hypothesis. Under the action of a proferment playing the rôle of a catalyzer, the autolytic diastases are activated and bring about their dissolving action.

We have already considered the principal objections which render this hypothesis untenable. It fails to explain serial action, for a proferment would disappear rapidly as a result of dilution in the course of passages; it does not take account of the fact that the bacteriophage presents itself in the form of autonomous particles capable of being counted; it is formally contradicted by the fact that the same bacteriophage can act on diverse bacterial species, etc. The inadmissibility of the hypothesis of Kabeshima has been recognized, moreover, by all authors who have considered the question.

REFUTATION OF THE HYPOTHESIS OF BORDET AND CIUCA

Bordet and Ciuca (October, 1920) very significantly modified the hypothesis of Kabeshima to the end of explaining serial activity. They said:

"In view of the fact that the stools of patients with dysentery are rich in leucocytes, and that the lysinogenic power is only observed toward the period of convalescence, we have asked ourselves if the phenomenon of d'Herelle is not the result of a defensive activity of the organism, and particularly of an activity of the leucocytic exudate. This produces in the bacterium an hereditary nutritive vitiation, consisting in the production by the bacterium of a sort of lytic ferment, which is capable, moreover, of diffusing in the ambient fluid and as a result, reacting in the same fashion on normal bacteria of the same species."

This proposition takes no account of the previously established facts. Among other facts it disregards that I had made it known some time previously that the bacteriophage was a normal inhabitant of the intestine, and that lysogenic power was also to

be observed at times other than at the moment of convalescence. At a time considerably earlier, I had likewise indicated that a strain of the bacteriophage exercised its action not only against a single bacterial species, but against several at the same time. Like the hypothesis of Kabeshima, this of Bordet and Ciuca takes no account of the fundamental fact, already demonstrated experimentally, of the existence of the bacteriophage in the form of particles which it is possible to count.

Fundamentally, how does this hypothesis of Bordet and Ciuca differ from that of Kabeshima? It differs in nothing except it be in the form in which it is stated. It hinges upon an arbitrary transposition of effect and cause; a transposition upon which they lay no stress. The leucocytic exudate induces the "nutritive vitiation" (?) which results in the lysis of the bacteria in the first tube of the series, but in the following ones the same effect will be produced, no longer by the leucocytic exudate, which will of necessity have disappeared in the first tubes because of the dilution, but by the bacterial lytic ferment alone. Bordet and Ciuca seem to find this substitution of cause entirely logical, when in reality it is contrary to all that we know. In admitting *a priori*, that a liquid, indeed a filtered liquid, is able to transport with it an hereditary property, there is, it appears, an affirmation which must needs be based on experiment and not solely upon the inference of Bordet and Ciuca. And what could have been the fundamental experiments which suggested such conclusions? They say:

> "If one or two days after the last injection, one removes by puncture the peritoneal exudate, rich in leucocytes, from a guinea pig which had received three or four intraperitoneal injections of *B. coli* at intervals of a few days, one can demonstrate that this exudate, when added to normal bacteria of the same species, modifies them, and confers upon them a very pronounced autolytic power, transmissible from culture to culture."

They add that they will shortly publish the results secured with other bacterial species. These results, promised more than a year and a half ago, have not yet been furnished. Furthermore, not having succeeded in reproducing the experiment with the leucocytic exudate, in spite of numerous attempts, I refuted the statements of Bordet and Ciuca, in a note published in the Compt.

rend. Soc. de biol. This contradiction has remained without a reply for more than eight months—evidence that these authors themselves have not succeeded in repeating the experiment.

Furthermore, the experiment with the leucocytic exudate, had it been correct, would in no case have provided proof for the non-reality of the bacteriophagous ultramicrobe, for it would not have disproved any of the experiments which demonstrate its reality, and of more significance, it accords perfectly with the idea of a parasitic ultramicrobe. Long before Bordet and Ciuca worked with the bacteriophage I had demonstrated that there was in certain cases a passage of the intestinal bacteriophage into the circulation, and that it could be isolated from the blood. Since the bacteriophage may acquire by adaptation the faculty of parasitizing any bacterial species, and since the bacteriophage is a normal inhabitant of the body of all animals, it is by no means impossible that one might experimentally succeed in provoking in the body of an animal this adaptation for a given bacterium which has passed into the circulation. We will see in Part II of this work that this is exactly the series of phenomena which occur in natural disease; and I can not see in what respect the fact of the experimental reproduction of this sequence of events will be opposed to the doctrine of an ultramicrobe parasitizing the bacteria.

I may say further, that even had their experiments been correct, the logical interpretation would not have been that of Bordet and Ciuca; that the bacteriophagous principle is derived from the leucocytes. In fact, if the *primum movens* of the bacteriolysis transmissible in series resides in the leucocytes, it should be enough, for example, to add to a suspension of *B. dysenteriae* the leucocytes from a horse furnishing an anti-dysentery serum, that is, from an hyperimmunized horse, to reproduce the phenomenon of lysis transmissible in series. This reaction would have been recognized long ago by innumerable investigators, perhaps first of all by Bordet, who for more than thirty years has investigated the antibodies in the blood of immunized animals. This experiment I have in vain attempted many times, well before Bordet and Ciuca announced their hypothesis, when I was attempting to test all possible hypotheses touching the origin

of the principle dissolving the bacteria. Far from possessing the ability to start serial lysis, the leucocytes derived from a horse hyperimmunized with the dysentery bacillus, do not even intervene to inhibit the growth of this bacillus, whatever may be the quantity of leucocytes employed in the test.

Finally, had the experiment of the leucocytic exudate been correct its interpretation would not in any case have served as a proof for the reality of an "hereditary nutritive vitiation" transmitting itself by means of a liquid factor. To be tenable, an hypothesis must take into account *all the facts*, and that of Bordet, exactly like that of Kabeshima from which it is copied, is incompatible with the experimental facts which I have reported. It implies, among other things, the strict specificity of the bacteriophage. Even if one admits as possible the entirely speculative hypothesis of "hereditary nutritive vitiation" transmitted by the intervention of a liquid, one is absolutely unable to admit that this liquid is able to transmit the "hereditary vitiation" from one bacterial species to another bacterial species. Moreover, Bordet and Ciuca at first maintained that there was such a strict specificity. However, in view of the accumulated evidence they have recognized that the action of the bacteriophage is not specific, but, in spite of this, they have not abandoned their conception or even offered anything by way of explanation.

The accidental positive result obtained by Bordet and Ciuca in the experiment with the leucocytic exudate can readily be explained in perfect harmony with the doctrine of the ultramicrobial bacteriophage. Such an explanation is, that the intestinal bacteriophage has passed into the peritoneal cavity of the experimental guinea pig as a result of the irritation produced there by the injections. This has been followed by a growth of the bacteriophage in this cavity at the expense of the bacteria injected. As we will see in Part II, the bacteriophage does not remain confined to the intestinal tract; it is able to enter the circulation. Moreover, the experiment of Bordet regularly becomes positive, even if the experimental guinea pig has received only one preliminary injection of bacteria, provided one or two cubic centimeters of a culture of the bacteriophage active for the bacterium inoculated is administered *per os* a few hours before the intra-

peritoneal injection. This experiment is adequate to give a correct interpretation to the accidental finding obtained by Bordet and Ciuca.

To summarize: the hypothesis of Bordet and Ciuca dealing with the nature of the bacteriophage is inadmissible, first, because it does not conform to the experimental facts; second, because it is founded upon an erroneous interpretation of an experiment which has not been repeated, and which, even if it had been correct, would not have served as a basis upon which such an hypothesis could be founded; third, because invoking as an explanation an hereditary phenomenon, it is in formal contradiction to all known facts concerning the hereditary transmission of characters.

Bruynoghe and his collaborators, who at the beginning of their studies adopted the point of view of Bordet, have since realized that the experimental facts do not harmonize with this hypothesis. They now support the idea of the ultramicrobe, a parasite of the bacteria.

REFUTATION OF THE HYPOTHESIS OF BAIL

Bail, in 1921, rejected the hypothesis of Kabeshima and of Bordet. According to him the bacteriophage existed indeed in the form of autonomous masses as I had demonstrated. It conducted itself as an ultramicrobe but these particles could only be constituted by the "splitter," that is to say, by particles derived from the lysed bacteria themselves. These organized particles, capable of reproduction under a filtrable form at the expense of the same bacteria, secreted a dissolving diastase.

Bail adduces in favor of his hypothesis the fact that he has been able to isolate from old cultures a bacteriophage active for the dysentery bacillus of Flexner. The cause of Bail's error is easy to detect. He has dealt with mixed cultures. I have already called attention to the frequency of such cultures and I have indicated their origin.

The hypothesis of Bail corresponds exactly to the second solution of the second possible hypothesis discussed above. It is needless to repeat the refutation which has been presented. Let us simply recall that the condition *sine qua non* for the validity

of this hypothesis would be strict specificity, and experiment demonstrates that this does not obtain. All authors are actually in accord on this point and have confirmed the observation that a single bacteriophage is able to attack diverse bacterial species.

REFUTATION OF THE HYPOTHESIS OF SALIMBENI

The hypothesis of Salimbeni is merely mentioned. Admitting that the bacteriophage is an autonomous microörganism, he considered it a Myxomycete, presenting itself in visible forms, and visible even to the naked eye. His observations have been contradicted by all who have studied the bacteriophage. Moreover, Salimbeni himself, has not continued to maintain this hypothesis. Possibly the observation upon which he based his hypothesis was due to the use of contaminated cultures.

CONCLUSIONS

All of the authors who have advanced hypotheses other than that of an ultramicrobe parasitizing the bacteria have forgotten that a hypothesis ought always to account for the entire mass of facts; that it is necessary, not only to demonstrate that it suffices to justify the phenomena, but it must also prove that these phenomena can not be justified if this hypothesis is abandoned or if it is modified.

After all, the whole controversy on the subject of the nature of the bacteriophage is only the renewal of the old discussion which we had for a long time thought terminated. With the new ideas of diastases capable of multiplication, or of co-ferments or catalyzers playing with the metaphysics of ubiquity, or of secretory immunity transmissible by communicated motion; it is only taking up anew the old theory of Stahl, that "any body brought to a state of putrefaction transmits very easily this state to another body still free of corruption." This theory of the multiplication of a principle of communicating motion had been held by Liebig in his discussion with Pasteur concerning the mechanism of fermentation. Pasteur demonstrated experimentally that it was false, and we were lead to believe the fact definitely acquired. With vital phenomena or communicating

motion, the discussion and the experiments of demonstration hinge on the same facts; we only descend a step in the order of magnitude of the beings concerned.

This same discussion may perhaps be renewed again some day if we descend still another step; if we discover, for example, a virus parasitizing the bacteriophage. The infinitely small is as conceivable as the infinitely great; we have not the right to assign limits to them.

PART II

THE RÔLE OF THE BACTERIOPHAGE IN IMMUNITY

INTRODUCTION

Up to the present time investigations on immunity have been directed toward solving the following question: What are the means of defense which permit an immunized animal or one naturally refractory to resist infection? These studies have resulted in the development of diverse theories.

When an animal is affected with a contagious disease of bacterial origin, the cellular immunity, which we call "organic immunity," abstracting it from all theory as to its intimate nature, is only established in a variable length of time after the inception of the disease. Does the animal remain without defense up to the time that this organic immunity becomes effective? By what phenomenon is it possible to acquire this organic immunity?

All animals sensitive to an infection and exposed to it do not contract the disease. Why do some of them remain unharmed?

These are the principal points upon which the experiments to be discussed have turned. As will be seen, they lead to a new chapter in the study of the means of defense against infection; and the conclusions themselves which will be derived from these investigations will not actually contradict anything in the present theories, for they apply to different states.

There is, nevertheless, a particular point in the present theories of immunity to which I wish to call attention, namely, that which deals with bacteriolysis as induced by specific sera. This is certainly pertinent since it deals with the subject under discussion:—the lysis of bacteria.

Everyone knows the nature of the phenomenon of Pfeiffer. If one injects a suspension of cholera vibrios into the peritoneal cavity of a guinea pig previously "immunized," a transformation of these vibrios into granules is noted. Pfeiffer has suggested that the transformation into granules constitutes only the first phase of the vibriolysis; but in this he was in error, for the granules maintain this form indefinitely.

We have very frequently sought for the act of disappearance of the granules in drops taken from the peritoneal fluid, but the number of these transformed vibrios has never diminished, even after several days, and we have therefore not been able to detect the phenomenon of dissolution of the granules. In spite of all this, it is incontestible that the granular transformation is a manifestation of a very grave change to which the cholera vibrios have been subjected under the influence of the peritoneal fluid of the immunized organism.[1]

Is this granular transformation, as Metchnikoff states, a manifestation of very grave lesions? The fact, established by him, that one of these granules seeded on agar gives a colony of normal vibrios is not an index of a very profound alteration. Let us consider for the moment that there can not be, in any sense, a lysis of the cholera vibrios in the Pfeiffer reaction. One may try by all sorts of methods to provoke the same phenomenon in all kinds of animals with all kinds of bacteria, but without result. It can only be secured with the cholera vibrios; they alone can be transformed into granules.

Metchnikoff, and later Bordet, showed that the reaction of Pfeiffer could likewise take place *in vitro*. To obtain a granular transformation it is only necessary to introduce the vibrios into the fresh serum of an immunized animal. Bordet further showed that this transformation was brought about by the interaction of two principles, amboceptor and alexin.

The amboceptor, thermostabile, is specific; that is to say, it is only active toward the element against which the animal furnishing the serum has been immunized. It exists only in traces, or not at all, in the serum of normal animals. It develops as an effect of immunization.

The alexin, thermolabile, is, it appears, common. It exists in as large an amount in a normal animal as in the immunized animal. It is fixed by any element previously acted upon by a specific amboceptor.

Bordet next discovered that the blood of an animal prepared by the injection of the red blood cells of a different animal species formed specific amboceptor. If to a suspension of these cells is added a heated serum of the treated animal (thus containing

[1] Metchnikoff. *L'immunité dans les maladies infectieuses*, Paris, 1901, Masson et Cie.

the amboceptor) and then some fresh serum from a normal animal (thus containing alexin) the phenomenon of hemolysis is obtained, —a phenomenon in which the dissolution of the red cells is simulated, although in reality, as Bordet himself showed, there is simply a diffusion of the hemoglobin, the stroma remains intact.

Finally, Bordet showed, in an indirect manner, by means of the complement fixation reaction which bears his name, that bacteria, as well as red blood cells, absorb specific amboceptor and are thus able to fix complement.

Here is where the equivocation commences. By analogy it was concluded that since, under the influence of the complement fixed by the cells in combination with the specific amboceptor, a hemolysis of the red cells was produced, so with bacteria which likewise absorb a specific amboceptor and fix complement in the same way, there *must necessarily* be a bacteriolysis. This bacteriolysis has never been observed directly, and this fact is adequate, it seems to me, to arouse some doubt concerning the reality of the phenomenon. The following experiment, which I have repeated several times, shows that in reality the bacteria are by no means destroyed under such conditions. The result is quite the opposite.

Experiment LVI. Take 4 tubes, each containing 20 cc. of 0.8 per cent saline. To each add a quantity of cholera vibrio suspension sufficient to give a count of about 1000 vibrios per cubic centimeter.

The first tube remains as the control.

The second tube receives 0.25 cc. of fresh guinea pig serum, after this serum has been shown by test to contain complement.

The third tube receives 0.1 cc. of an anticholera serum, in which the presence of specific amboceptor has been demonstrated.

The fourth tube receives 0.25 cc. of the fresh guinea pig serum and 0.1 cc. of the anticholera serum. Thus, in this tube, the vibrios are in the presence of a specific antibody and of complement. The 4 tubes are incubated at 37°C., and from day to day are tested to see if the vibrios are alive or dead. To this end, after twenty-four hours, the tubes are thoroughly shaken and from each of them 2.5 cc. is immediately taken and planted into a tube of sterile bouillon. The first of the tubes usually (4 times in 6) remains sterile[2] while the other three give cultures of the cholera vibrio,

[2] This bactericidal action of physiological saline is rather strange. Even when working with relatively concentrated suspensions, containing from 50 to 100 million bacteria (cholera vibrios or *B. dysenteriae*) per cubic

normal in the second tube containing complement only, agglutinated in the last two.

After forty-eight hours transfers are again made. The first tube always remains sterile, the second is often sterile (3 times in 6), the last two give agglutinated cultures.

After three days the subcultures result as follows; the first two tubes are always sterile, the third often so (4 times in 6), the last always gives an agglutinated culture.

After four days the first three tubes are always sterile. The last tube only, that is, the one containing both antibody and complement, gives an agglutinated culture.

After six days the same result is obtained.

The same experiment has been performed with the Shiga dysentery strain and the anti-dysentery serum of the Pasteur Institute. The result was in all respects comparable. The Shiga bacillus, like the cholera vibrio, persists for a long time in the suspension containing the anti-serum and the alexin.

Variations in all directions have been made in the proportions of the sera, both in that containing the complement, and in that containing the antibody, as well as in the concentration of the bacterial suspension. The results have all been essentially the

centimeter, the sterilization is complete in a few hours at a temperature of 37°C. On the contrary, these organisms will remain alive for some days in tap water. And what is still more singular, is that everyone has adopted physiological saline for the preparation of bacterial suspensions, concluding *a priori*, that bacteria *must* be preserved alive for a long time in a medium spoken of as "isotonic."

At the bottom of this we find a false deduction by comparison. Red blood cells hemolyze in a few seconds in tap water, but, on the contrary, they resist hemolysis in isotonic saline solution. Thus, it is concluded, without doubt, that bacterial cells *must* conduct themselves in the same manner. As a matter of fact, this is absolutely contrary to what takes place.

As we will see, the same reasoning has been held with regard to the so-called bacteriolysis with sera. There also, one falls into an error, and this will always be the case when an attempt is made to substitute deduction based on analogy for experimentation, especially when the elements concerned are as different as a bacterium and a red cell.

With reference to the toxic action of sodium chloride, Loeb (Biochem. Zeitschr., 1906, **2**, 81) has shown that this salt may be toxic for all the unicellular organisms living in the sea, and that this toxicity may be neutralized by the salts of potassium and calcium.

same. The bacteria always remain alive in the antibody-complement mixture for a much longer time than in pure physiological saline.

These experiments show that cholera vibrios, or dysentery bacilli, are rapidly destroyed in saline; that they remain alive longer in the presence of a normal fresh serum containing complement or in an antiserum; and that they remain alive still longer in the presence of both the antiserum and complement. It is therefore evident that the bacteria, sensitized and having fixed complement, far from being subjected to lysis, are more resistant than normal bacilli.

The sera termed "antibacterial" do not, *in vitro* at least, play any bacteriolytic rôle. Everything indicates that it is the same *in vivo*. We know that at times the antisera, derived from horses thoroughly immunized by the injection of living bacilli, are contaminated by bacilli of the same species as those which have been injected, and that it is possible to demonstrate them by culture. Anti-rouget serum provides a remarkable example. The serum from a horse hyperimmunized by a series of injections of living culture possesses extremely marked curative and preventive properties, but it is, nevertheless, rather frequently contaminated by the bacillus of rouget, even if the serum is withdrawn some ten to twelve days after an injection. How can we reconcile this fact, which indeed is not an isolated observation, with the hypothesis that the immunity which it confers is, by some mechanism at present unexplained, associated with the presence of an "antibacterial antibody?" If it should produce bacteriolysis, it certainly ought to do so in the hyperimmunized animals themselves, where the bacterium finds itself in contact with an abundance of antibody and of complement.

On the other hand, sera very rich in antibody may entirely lack preventive power, and the opposite is also true. Metchnikoff and his collaborators have furnished many examples of this.

If we pass to a consideration of natural immunity we readily discern that there is absolutely no parallelism between the state of the patient and the antibody content of the blood. In human typhoid fever, for example, the fatal relapses may occur when the antibody is at its maximum. Finally, in diseases which result

in immunity the antibody disappears a short time after the attack, but this does not prevent the persistence of immunity for years, or even decades, after the disappearance of the antibody.

To summarize: however the serum of hyperimmunized animals or the serum of an individual affected with some infectious disease may act, it is impossible to establish any relation between the antibacterial antibodies—so-called—and immunity. These antibodies, like the agglutinins, can only be considered as indices of infection.

It may be objected that preventive vaccination by a sensitized virus indicates a fragility in the bacteria impregnated with antibody. This objection is in reality not an objection, for experimental facts show, on the contrary, that a sensitized bacterium is as virulent as a normal bacterium. I have proved this for *B. typhi murium* with the mouse, and for the bacterium of bovine hemorrhagic septicemia in cattle. These animals are killed in the same time and by the same dose, whether the bacteria injected are normal or sensitized. The possibility of injecting man, without great inconvenience, with sensitized cholera vibrios or sensitized typhoid bacilli (and it may still be said with some reserve in this last case) does not negative these findings at all, seeing that Ferran has given tens of thousands of preventive vaccinations against cholera, using living cultures, and that Ch. Nicolle has demonstrated the possibility of vaccinating man against typhoid fever by injecting him with living, normal typhoid bacilli. In general, injections of sensitized bacteria are no more inoffensive than injections of the normal living organisms, and they are equivalent, since living sensitized bacteria and living normal bacilli are virulent to the same degree.

One cannot avoid the conclusion that it is impossible to attribute any active rôle in the production of antibacterial immunity to any actually known antibodies. All *organic* immunity is reduced to antibacterial immunity, assured by phagocytosis, and to antitoxic immunity, assured by the antitoxins.[3]

But is this organic immunity an attribute of the immunized animal only? Is it not enjoyed by a susceptible animal? All

[3] Meaning by antitoxins all antibodies which neutralize a soluble toxic substance.

individuals exposed to an infection do not contract the disease, and to what do they owe this privilege? Once diseased, the immunity in the susceptible animal manifests itself only after a lapse of time, which in the most favorable cases is hardly less than twelve days.[4] Does the animal remain without defense during this lapse of time? Finally, in this animal affected by disease, whatever may be the mechanism of organic immunity, how can this immunity be established to render this sick animal refractory? Under what influence is phagocytosis released? Under what influence do the antitoxins originate?

The rôle which the partisans of the theory of "bactericidal humoral immunity" have desired that these simple indices of infection—the antibodies—play, is based upon a non-existent phenomenon of bacteriolysis by immune sera. Could it not in reality be played by the bacteriophage, that principle endowed with a powerful bacteriolytic action, operating upon the most varied bacteria?

Could not the bacteriophage play a rôle in the defense of the organism, a preponderant rôle in the susceptible animal, and as such, deprived of all acquired immunity? In other words, does there not exist by the side of the homogeneous organic immunity, an immunity originating in the bacteriophagous ultramicrobe, and, as a result, an heterogeneous immunity?

We have seen in a preceding chapter that the lysin of the bacteriophage may possess an extraordinarily potent opsonic activity. Can not the bacteriophage play, in addition to its direct action, an important rôle in phagocytosis itself, in bringing about what might be called a phagocytic education?

[4] Animals vaccinated by an attenuated anthrax or rouget virus are not protected against natural infection until after this period of time. I have also shown that at least twelve to fifteen days are necessary to secure an immunity following the use of an attenuated virus in bovine homorrhagic septicemia. In typhoid fever, the immunity acquired as a result of infection is even longer in establishing itself, as the possibility of relapse in well-advanced convalescence shows. This lapse of time, twelve days, is therefore a minimum insofar as naturally acquired organic immunity is concerned. There is nothing to be gained here by discussing laboratory experiments concerning the development of immunity in refractory animals, for such experiments are laboratory phenomena only and have nothing necessarily in common with natural conditions.

Finally, in dissolving the bacteria, can it not be an indirect factor in naturally acquired antitoxic immunity?

These are the points which we will consider in the second part of this monograph.

It would seem that the only method which ought to be followed in investigating the relation between immunity and a principle to which one may attribute a protective power ought to be founded on the observation of natural disease, and that the parallelism between the state of the patient and the presence, and the potency, of the supposed protective principle, ought to serve as the criterion for determining its true rôle. If a parallelism exists, it may be regarded in the possible relation of cause and effect, and one can then turn to the counter-test for confirmation. If a parallelism does not exist, the relation of cause and effect cannot be invoked, and the principle under consideration cannot play an active rôle in the processes of recovery. This is the method of investigation, the only logical one it seems to me, that I have applied in investigating the relationship between the bacteriophage and immunity. I consider, in fact, that a theory of immunity based only on simple observation or on comparison, always remains subject to discussion. For simple observation readily leads to error, especially when the observations are made on refractory animals and are not found to be confirmed when applied to a susceptible animal. It is certainly much easier to experiment in the laboratory with caged animals; to study the immunity against the cholera vibrio, for example, on a guinea pig which is resistant to the disease naturally, than to run everywhere in search of epizootics in order to study the disease in its normal environment. But common sense alone is adequate to make it apparent that the first method can prove nothing, and that only observation of the natural disease, complemented by experimentation on an animal susceptible to it, can give results that have an absolute value. It may indeed seem strange that we use the word "immunization" in speaking of a refractory animal, since the refractory state already represents immunity carried to its highest degree.

I wish to be free of such criticism and will thus follow an order which seems to me the most logical. We will observe first,

natural infection and we will see if the search for the bacteriophage and the determination of its properties at different stages of the disease and of convalescence provides results which have any relation to the pathologic condition of the patient. I have selected for this investigation different infections, enteric and septicemic, diseases of man and of animals, with which we will show that defense by the bacteriophage is a phenomenon of a general nature. Some of the diseases studied are epidemic, and we will have occasion to note the effect of the bacteriophage on the progress of the epidemic itself.

If the bacteriophage is an agent of immunity, it will not appear only at the exact moment when it is most needed. It should be a normal inhabitant of the intestine. We will look for it, then, in the healthy individual, choosing subjects throughout all animal species, and this will show the generality of the presence of the bacteriophage.

Finally, we will attempt the counter-test. If, in the susceptible animal the principle of antibacterial immunity resides in the bacteriophage, the administration to a susceptible animal of a bacteriophage active for a given bacterium ought to render the organism resistant to the disease caused by this bacterium.

Thanks to the kindness of M. Roux, Director of the Pasteur Institute, and to M. Yersin, Director of the Pasteur Institute in Indo-China, I have been able to accomplish in its entirety the program which I have outlined.

In France I have had the opportunity to study the rôle of the bacteriophage in intestinal diseases, and during the course of a year spent in the Pasteur Institute at Saïgon, I have been able to verify the generality of the phenomena observed, by a study of a highly contagious septicemia,—barbone in the buffalo,—and by a disease of glandular localization,—plague.

It is certain that a theory of immunity based on the bacteriophage, that is, on an autonomous organism, is so far outside of all present opinion that it will stir up at first incredulity and will be called a "finalistic theory,"—a synonym of "anti-scientific." I affirm that from my point of view this theory can not be "finalistic." "To be is to struggle, to live is to conquer," a very just statement by Le Dantec. It is all contained in a single word—

evolution. A being which evolves is necessarily a being which lives, which adapts itself, and which conquers. From the instant that it ceases to adapt itself—to evolve—it dies. Evolution is always conducted according to the law of least effort. The multicellular organisms have profited by securing for their defense the parasitism of the bacteriophage for the bacteria; which is only a chapter in the universal struggle.

If, among all living beings, the bacteria alone escaped parasitism, where would we arrive? It is very simple. One of two things would take place. Either evolution would not extend beyond the stage of the unicellular being, or evolution would be accomplished in another manner and immunity would be assured by other means; a simple matter of adaptation. The bacteriophage does not exist for the defense of the superior organism against the bacteria, it exists simply because in the course of evolution certain germs have parasitized others.

Nothing in nature exists simply for an end, for nature is not an end. That there exist on the earth thinking beings, or that they might not have been, is a perfectly negligible incident. Is this point of view "finalistic"? But what does it matter; a scientific theory is true or false according to the proofs upon which it is founded.

Each time that we will speak in the course of this discussion of "antibacterial immunity" it is essential to understand "antibacterial immunity in a susceptible individual." These observations and experiments, as I have already remarked, are concerned with this and this alone.

Up to the present I have paid little attention to the phenomena of immunity in the refractory animal. It indeed seems, in general, in the special type of immunity which characterizes the refractory state, that the elimination of bacteria which may gain access to the body, and which because of the refractory state are not pathogenic, is effected by phagocytosis. In this special case, defense by the bacteriophage could not possess, the greater part of the time, the opportunity to act. Phagocytosis is accomplished too rapidly to allow the bacteriophage time to increase its virulence toward the bacterium which is introduced into the organism.

CHAPTER I

The Bacteriophage in Disease

Choice of Diseases to Study. Bacillary Dysentery. *B. coli* Infections. Typhoid and Paratyphoid Fevers. Avian Typhosis. Barbone. Bubonic Plague. Flacherie. Conclusions.

CHOICE OF DISEASES TO STUDY

From the point of view of the study of immunity human infection offers an inconvenience. Man is not available for experimentation; observation alone is permitted. On the other hand, the study of a human infection, such as typhoid fever or cholera for example, in a refractory animal—and they are all so—can only lead to illusory results. Study of disease in the animal, on the contrary, permits of confirmatory experimentation upon the susceptible animal itself where error is no longer unavoidable. However, this method of procedure is very complicated; the disease does not come to us, we must go to it.

The study of typhoid fever and of dysentery allows us to show by observation the rôle of the bacteriophage in the course of the disease. These same phenomena may be reproduced in the course of infection in animals, and it is possible with the latter to conduct such experiments of verification as will confirm that which simple observation has already shown.

In order to ascertain the influence of the bacteriophage on the morbid state, a method which consists in investigating at random the activity of the bacteriophage in a specimen of material taken at any time whatsoever will not lead to any result. It is necessary to take the patient as quickly as possible after the inception of the disease and to examine the feces each day until recovery is complete. The daily findings are then plotted in a curve which is superimposed on that expressing the general state of the individual, such as the number of stools in dysentery, or the temperature in typhoid. A comparison of these two curves allows one to draw a conclusion. This mode of procedure necessitates

considerable work, but it must be applied for it is the only procedure which will allow of a conclusion.

We have seen in the course of the preceding chapters that the virulence of a strain of the bacteriophage is rarely limited to any one particular bacterial species, but exercises in general, with variable intensity, its action on several species pertaining to the same group or to closely related groups.

It would be practically impossible, in view of the length of the operations, to investigate all of the bacteria which may be attacked by a bacteriophage isolated from the stools of a patient at any given time. The procedure must, therefore, be reduced to a systematic examination in each case of the virulence of the bacteriophage toward the particular bacterium involved in a causal relationship. The virulence should be determined for a type strain of this bacterium which has been cultivated for a long time in the laboratory, for the strain derived from the patient himself, and for *B. coli*. Eventually, the investigation may be extended to bacteria belonging to the same group or to related groups.

We know that the virulence of different strains of bacteriophage for a given bacterium is far from constant. It varies throughout a scale which goes from zero to an activity such that it is sufficient to add only a few germs to a heavy suspension of this bacterium in order to obtain within three or four hours a complete and permanent lysis, all the bacteria being then destroyed. Between these two limits,—no virulence and extreme virulence—all intermediate degrees are possible. A weak virulence we have seen, may be enhanced *in vitro*, but in so far as the study of immunity is concerned, the point in which we are interested is the virulence presented by the bacteriophage in the organism at the moment of observation; or the actual virulence of the bacteriophage contained in the filtrate prepared directly from the feces at any given time during the disease.

As we have also seen, the appearance of cultures of the bacteriophage in bouillon or on agar enables us to evaluate its virulence for the bacterium in question. In order to facilitate explanation in the further discussion of the subject we will adopt a scale of virulence fixed as follows:

0 = no virulence toward a given bacterium. Normal cultures of the bacterium develop in bouillon or on agar, whatever the quantity of the filtrate from the feces which had been added.

+ = weak virulence. The growth in bouillon of the bacterium to which the filtrate has been added is apparently normal. Transfer of this culture to agar gives, after incubation, a culture layer showing a few minute plaques. Some of the bacteriophagous germs have therefore attacked the bacteria and have formed colonies.

++ = medium virulence. The culture of the bacterium to which the filtrate has been added is almost normal in bouillon. Transfers of this culture to agar give, after incubation, either a culture layer of the bacterium studded by very numerous colonies of the bacteriophage, presenting an appreciable surface area, or of fragments of bacterial culture because of the very great number of bacteriophage colonies.

+++ = high virulence. Lysis of a bacterial suspension is obtained but secondary cultures constantly develop. The reinoculations on to agar remain sterile or give only rare colonies of the bacterium.

++++ = extreme virulence. The bouillon suspension shows complete, and, in general, permanent lysis. Inoculations on to agar always remain sterile.

Obviously, it would be possible to establish a more detailed scale of virulence. (Moreover, this has been done in the curves which will be given, where the interval between no virulence and extreme virulence has been subdivided into ten steps, in accordance with the aspect of the cultures, the number of colonies of the bacteriophage, and the size of the plaques, which bear a relation to its virulence.) Practically, the appreciation is adequate with four steps, particularly in view of the fact of the extreme variability of virulence in the bacteriophage in the body of a single individual from one time to another.

The expression "Shiga ++++, Hiss +, Flexner 0, Typhoid ++, Para A 0, Para B 0, *B. coli* +++" means, then, that the bacteriophage contained in the filtrate derived from the stool of an individual presents an extreme virulence for *B. dysenteriae* Shiga, a weak virulence for *B. dysenteriae* Hiss, an average virulence for *B. typhosus*, and a high virulence for *B. coli*, with none for *B. dysenteriae* Flexner or for the paratyphoids A or B.

BACILLARY DYSENTERY

The subjoined curves show, much better than any explanation, the relations which exist between the condition of the patient and the virulence of the intestinal bacteriophage against this pathogenic bacterium. The upper tracing gives the number of stools in 24 hours; the single line indicating stools without blood, the double line those containing blood and mucus. On the lower portion of the chart is indicated (*1*) by the dotted line, the virulence of the bacteriophage for the colon bacillus; (*2*) by the broken line, the virulence of the bacteriophage for the stock strain of the Shiga bacillus which had been maintained for a long time under laboratory cultivation; and (*3*) by the heavy line, the virulence for the Shiga strain taken from the patient himself.

The five cases given as examples have been treated at the Pasteur Hospital. It has thus been possible to follow them with all necessary attention and to obtain material for examination as often as the investigation demanded; at least once, often several times, during the course of each day.[1]

For these examples, cases of different severity have been selected. In all of them *B. dysenteriae* Shiga was isolated from the stools at the beginning of the disease.

1. Germaine Mel. . . . (sixteen years, fig. 1). This was a mild case of dysentery. The patient was an inmate in an institution where there were about thirty young girls. During the period from the 12th to the 22nd of July about twenty of these girls presented intestinal disturbances of sudden onset, accompanied by a profuse diarrhea, followed by a rapid amelioration

[1] I must here thank the sisters, nurses in the Pasteur Hospital, who have with unwearying kindness provided me with the numerous specimens which have allowed me to follow the condition of the patients.

of symptoms. Within one or two days after the onset all had again become normal. In only one or two cases did the stools contain traces of blood. In order to establish a diagnosis the directrix was asked to send a patient to the Hospital during the earliest symptoms.

Germaine Mel. . . . entered the Hospital on the 18th of July. From the first stool passed after her arrival a bacillus presenting the biochemical characteristics of the Shiga bacillus was isolated

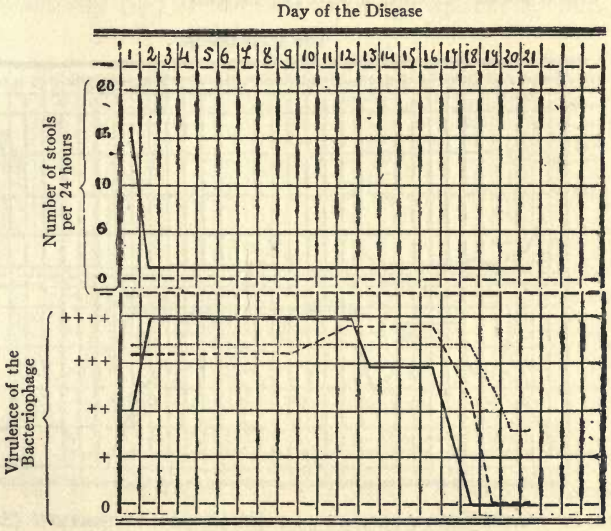

FIG. 1. GERMAINE MEL. . . . DYSENTERY (SHIGA)

Virulence for { *B. dysenteriae* from the patient———
B. dysenteriae, stock strain - - - - - - -
B. coli.....

after considerable difficulty. It was inagglutinable, and it was only after three passages on agar that agglutination was secured (1:500).

As can be seen in the tracings, the number of fluid stools, seventeen on the first day, fell quickly during the second day to two, without medication.

The intestinal bacteriophage, isolated from the fifth stool of the first day, was endowed with an extreme virulence for the bacillus

of the infection, and with a somewhat lower grade of virulence for the stock Shiga strain and for *B. coli*.

The stools of eleven of the inmates of this institution were examined. Among the number were nine who had shown intestinal disturbances two or three days previously. Two had shown no morbid symptoms. All of those examined contained a bacteriophage with a high or extreme virulence for the Shiga strain isolated from the stool of Germaine Mel. . . . as well as for the stock strain of Shiga and for *B. coli*.

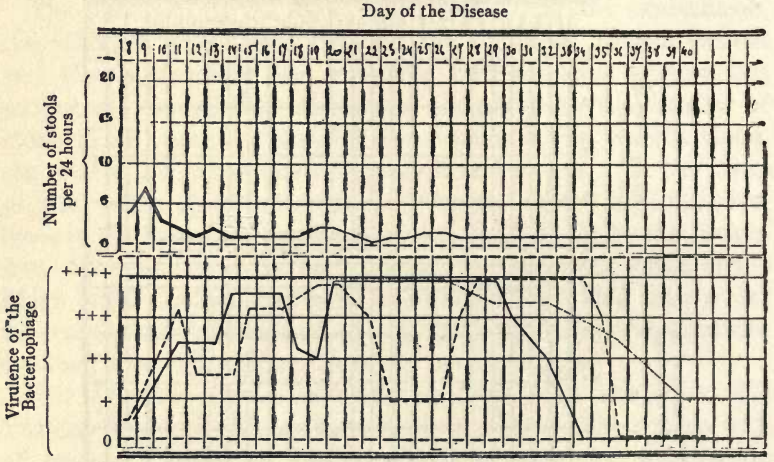

FIG. 2. MARIE LEB. . . . . (26 years) DYSENTERY (SHIGA)

Virulence for { *B. dysenteriae* from the patient ——— *B. dysenteriae*, stock strain - - - - - - - *B. coli*. . . . . }

═══ Stools contained blood

Therefore, with Germaine Mel. . . . there was a bacteriophage of maximum activity, even from the beginning of the disease. Recovery took place within twenty-four hours.

2. Marie Leb. . . . . (twenty-six years, fig. 2). This case was one with a mild dysentery, due to *B. dysenteriae* Shiga. The stools were typical, containing blood and mucus. Entrance to the Hospital took place on the eighth day of the disease. The first stool containing blood had been passed the day before.

Upon entrance to the Hospital the feces contained a bacterio-

phage active for the Shiga organism (+), extremely active for *B. coli* (++++), and but very slightly active for the dysentery bacillus found in the patient (+). Against this last bacillus the virulence increased during the course of the three following days, reached its maximum activity (++++), fell away somewhat (++), and then definitely regained its full virulence (++++). These fluctuations in virulence were reflected in the condition of the patient. At the end of convalescence there remained only a slight activity (+) of the bacteriophage against *B. coli*.

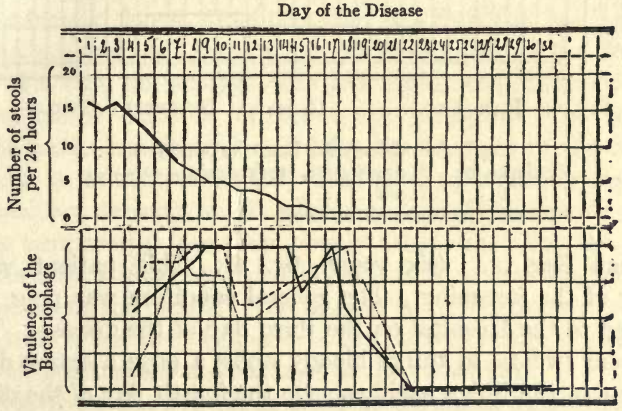

FIG. 3. VICTOR KER. . . . . (6 years) DYSENTERY (SHIGA)

Virulence for { *B. dysenteriae* from the patient———
B. dysenteriae, stock strain - - - - - - -
B. coli. . . . . }

═══Stools contained blood

3. Victor Ker. . . . (5 years, fig. 3). The dysentery was due to the Shiga bacillus, was of moderate severity, and was contracted by contact with the patient next discussed. When admitted to the Hospital, on the third day of the disease, the intestinal bacteriophage already manifested an average virulence (++) for the stock Shiga strain as well as for the strain isolated from the patient. This virulence increased rapidly and maintained a high value up to the time of complete convalescence (+++). It then abruptly disappeared.

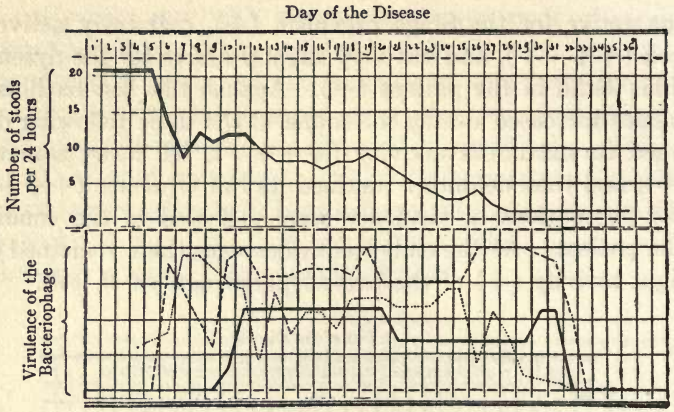

FIG. 4. JEAN KER. . . . . (6 years) DYSENTERY (SHIGA)

Virulence for { *B. dysenteriae* from the patient ——— / *B. dysenteriae*, stock strain - - - - - - - / *B. coli*. . . . . }
═══ Stools contained blood

4. Jean Ker. . . . (six years, fig. 4). This patient was a brother of the foregoing. The general condition was poor when admitted to the Hospital on the third day of the disease. There were from twenty to thirty bloody stools a day; a severe dysentery due to the Shiga bacillus. On the fourth day of the disease there were twenty-four bloody stools. The bacteriophage was feebly active (+) for *B. coli* and was inactive for the Shiga bacillus. The record shows the following:

DAY OF DISEASE	NUMBER OF BLOODY STOOLS	VIRULENCE OF THE BACTERIOPHAGE AGAINST		
		B. dysenteriae (patient)	*B. dysenteriae* (stock)	*B. coli*
5th	23	0	+	+
6th	13	0	++++	++
7th	9	0	+++	++++
8th	12	0	++	++++
9th	11	0	+	++++
10th	12	+	++++	+++
11th	12	+++	++++	+++
12th	(4 of 6 stools without blood)	+++	++++	+

From this time on improvement became more and more marked. The activity of the bacteriophage did not disappear after convalescence had been established.

In the first three of these cases the dysentery was mild. The bacteriophage was active at the onset, the bacterium did not acquire a resistance, and its growth was quickly suppressed. In the last case there was a struggle and the bacillus acquired a resistance which was finally overcome. The condition of this patient was much more serious.

5. Lans. . . . (seventy years, fig. 5). This case illustrates an extremely severe dysentery due to the Shiga bacillus. The patient entered the Hospital on the second day of the disease.

In this case the struggle was prolonged, with fluctuations due to the mixed cultures formed in the intestine. The condition of the patient registered faithfully the changes in the struggle. It may be noted particularly that the bacteriophage manifests a transitory activity on the eleventh day of the disease and the stools temporarily lose their bloody character. But the bacillus increases its resistance and this permits it to develop, and blood reappears in the stools. The disease is only definitely overcome at a time when the virulence of the bacteriophage is sufficiently high to dominate the resistance of the bacterium.

Aside from the five cases cited as examples others have been followed, both in France and in Indo-China. Seventeen other cases differing in severity were examined daily, and twenty-nine more were observed less frequently. In all of the cases the activity of the bacteriophage was manifested in an identical manner:

1. In case of recovery, the virulence of the bacteriophage commences to manifest itself in a marked manner toward *B. coli.*

2. The virulence next extends to the type strain of the Shiga bacillus, that is to say, toward a strain which has been for a long time under artificial cultivation and which, for this reason, has been deprived of much of its resistance.

3. It manifests itself next, more or less quickly, toward the Shiga bacillus isolated from the patient himself at the onset of the disease.[2]

[2] Obviously it is necessary to preserve this strain without replanting. The isolated colonies obtained on the original plates are planted on several

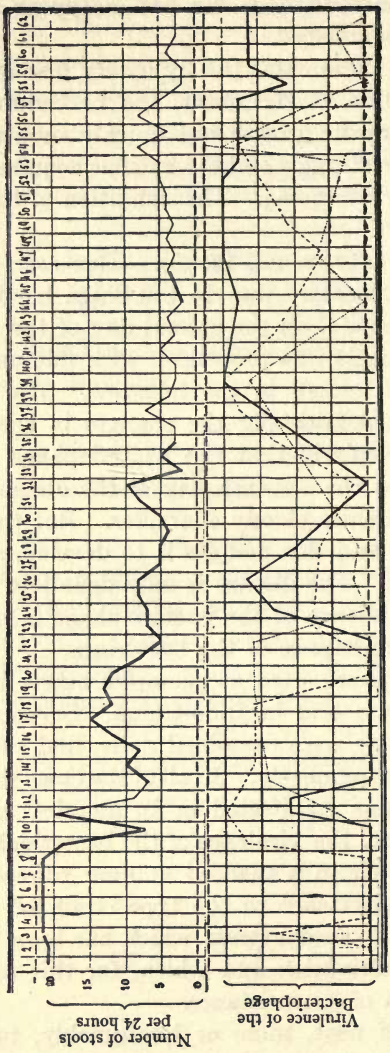

FIG 5. LANS. . . . . (70 years) DYSENTERY (SHIGA)

Virulence for { *B. dysenteriae* from the patient ——— *B. dysenteriae*, stock strain - - - - - - - - *B. coli*. . . . . ═══ Stools contained blood

4. In all cases the fluctuations in the virulence, as well as the fluctuations in the resistance of the bacteria, parallel the state of the patient, and the onset of improvement coincides with the moment when the virulence of the bacteriophage dominates clearly the resistance of the bacterium. We thus see reproduced *in vivo* the same mode of action as that observed *in vitro;* permanent and complete lysis, mixed cultures with negative transfers, mixed cultures with alternations in the dominating force.

In Indo-China an opportunity was afforded to follow four fatal cases of bacillary dysentery in natives. At no time during the course of the infection did the intestinal bacteriophage show a trace of activity for the Shiga bacillus, either for the stock strain or for that isolated from the stools of the patients.

A last case offers an especial interest, for it shows that it is not only *in vitro* that the bacteria are able to become refractory to the action of the bacteriophage. Although this may occur *in vivo* these cases must be very rare, even exceptional.

Alix Desp. . . . (fifty-six years). The patient entered the Pasteur Hospital on September 26, 1919. At the time of admission there was a profuse mucous diarrhea with thirty to forty stools a day. Examination of the intestinal contents gave an almost pure culture of a dysentery bacillus presenting atypical characters, as follows:

Non-motile bacillus. Gram negative. Indol positive. No blackening of lead acetate agar. No change in neutral red media. Litmus sugar agar media not fermented with any of the sugars. In Barsiekow's medium, maltose and lactose are unchanged, glucose and mannite are turned red. After six transfers on agar it agglutinated to the titre (1:6000) with a Hiss agglutinating serum, to 1:400 with an anti-Flexner serum of which the titre was 1:6000, and was not agglutinated at 1:20 by an anti-Shiga serum.

In spite of these atypical characters it is, then, a Hiss strain possessing weak fermentative properties.

When secured from the body this bacillus was not affected by a bacteriophage very virulent for a normal Hiss bacillus, but it

agar tubes and a portion is taken from these tubes for the tests conducted during the course of the disease. It is well-known that resistance is attenuated by successive transplantations.

was lysed after about a dozen transplantations. It was, then, a bacillus which was refractory to the bacteriophage when taken from the body.

At the same time a strain of bacteriophage was isolated from the stools of the patient. This presented the following virulences: Shiga 0, Flexner ++; stock culture strain of Hiss ++++; *B. coli* +; the Hiss strain from the patient +. After twelve subcultures of the Hiss strain from the patient the virulence of the filtrate was again tested. Perfect lysis was secured, showing that the bacillus had lost its resistance by transfers on agar.

The bacteriophage of the patient is active to a maximum degree against a stock strain of the Hiss bacillus but it is only slightly active for the individual strain causing the infection, with which it forms, *in vitro*, mixed cultures indefinitely cultivable. There was likewise in the intestine of the patient a mixed culture of the bacteriophage and the refractory Hiss strain.

In spite of every care and repeated injections of anti-dysentery serum the patient became more and more weak; the temperature oscillated between 38° in the morning and 40°C. in the evening; the number of stools gradually increased and became uncountable on about the thirtieth day; and at about this time the patient fell into a marasmic condition, the temperature stayed at about 38°C. and death occurred on the thirty-fifth day.

Bacteriologically, the stools, tested each day, showed an almost constant bacterial flora. The pathogenic bacillus was always abundant, often in almost pure culture, and presented the characteristics described. The virulence of the bacteriophage increased continuously until the fifteenth day when it became fixed, showing:—Shiga ++++; Flexner ++++; Hiss ++++, *B. typhosus* +++; *B. paratyphosus A* +++; *B. paratyphosus B* +++; *B. coli* ++++; bacillus of the patient 0 (completely refractory) when freshly isolated, +++ after fifteen transplants.

At autopsy[3] there was isolated from the contents of the colon, from a fragment of mucous ulceration, from the liver, from the spleen, and from the heart blood, a Hiss dysentery bacillus, presenting the same characteristics as that which had been isolated

[3] Performed by L. Géry, whom I thank for the specimens he was kind enough to send me.

at the beginning of the disease. From all the organs a bacteriophage was isolated presenting the same characters as that which had been isolated from the stools and whose virulence has been indicated.

This case, altogether exceptional (I believe that it is the first case reported of a *B. dysenteriae* Hiss septicemia) is very interesting for it shows in an unquestioned manner the rôle that the bacteriophage plays in the defense of the organism. In all of the cases examined heretofore we have seen, either recovery starting from the time when the bacteriophage had acquired sufficient virulence to dominate the pathogenic bacillus, or death in the case of the failure of adaptation. In this last case, the bacteria developed a refractory condition, the bacteriophage was overcome and remained without action whatever its virulence may have been. The barrier thus being lacking, the bacteria developed freely and invaded the entire organism. The patient succumbed to a septicemia with the Hiss bacillus.

This exceptional case provides us with new information. A bacterium is pathogenic for a given organism if it secretes substances toxic for the cells of this organism. It is the more virulent the more capable it is of development at the expense of this organism. The dysentery bacilli are pathogens because of this secretion of toxic substances, for they do not invade the organism, but remain localized in the intestine and in the intestinal mucosa. Nevertheless, in the case of the woman Desp. . . . the Hiss strain was accidentally endowed with an extreme virulence, and this solely because the bacteriophage had been overcome. This suggests an idea which we will have occasion to confirm in the following chapters,—that the virulence of a bacterium at any given moment is the greater if its resistance to the bacteriophage is at this time high.

The case Desp. . . . is exceptional. As a general rule death occurs in dysentery, not because of the acquisition by the bacterium of a refractory condition, but by a failure of the bacteriophage to adapt itself to bacteriophagy toward the pathogenic organism. In the four cases mentioned above which were fatal, a bacteriophage active for the Shiga bacillus could not be isolated at any period of the disease.

During the course of the epidemic of dysentery which occurred in the region of Paris during the early autumn of 1918, an opportunity was given to observe twenty-nine cases of benign diarrhea. In all of these cases a bacteriophage of very high or extreme activity for the Shiga bacillus was isolated from stools taken the day after the malaise. This bacillus was the cause of all the severe cases studied at this same time.

Living at this time in a locality (Meulan) where several severe cases of dysentery were noted together with a large number of cases of transitory diarrhea, I examined the stools of nine persons who were healthy, but who lived in contact with individuals who had had dysentery. From these nine individuals a bacteriophage of average or high activity for the Shiga bacillus was isolated. We have noted above that the same fact was observed in the institution where Germaine Mel. . . . had contracted dysentery. Individuals who are exposed to infection and who resist show therefore in their intestine a bacteriophage virulent for the causative pathogenic bacillus, exactly like the affected individuals who recover.

As a result, in an epidemic period the simple cases of diarrhea must in reality be cases of aborted bacillary dysentery, thanks to the rapidity with which the intestinal bacteriophage adapts itself to bacteriophagy against pathogenic bacteria. And healthy individuals, living in contact with affected people, are only spared by virtue of a still more rapid adaptation occurring before morbid symptoms appear. We will find comparable facts in all the diseases which we will discuss.

To summarize: the pathogenesis and the pathology of bacillary dysentery are dominated by two factors, operating in different directions; the dysentery bacillus as the pathogenic agent and the bacteriophage as the agent of immunity. The history of a case of dysentery is only the story of the struggle, occurring within the body, between these two factors, and the condition of the patient faithfully reflects the vicissitudes of the struggle.

In case of a rapid enhancement in the virulence of the intestinal bacteriophage toward a pathogenic bacillus, the latter is unable to develop a resistance and is destroyed in the struggle, so that the disease aborts before the appearance of any symptoms or manifests itself only in a transitory disturbance.

The increase in the virulence of the bacteriophage for the invading bacterium may be retarded for one of two reasons:—First, as a result of unfavorable intestinal conditions. (We have seen the considerable importance, *in vitro*, of very slight variations in the reaction of the medium on the development of the ultramicrobial bacteriophage.) In accordance with the chemical and physical state of the intestinal contents, one bacterium is favored at the expense of another; the intestinal fermentations, and as a result, the reaction of the medium will vary according to the predominating flora. The development of the bacteriophage is then doubly influenced, first, by a change in the state of the medium itself, and second, by changes in the flora which increase or decrease, according to circumstances, the bacterial species at the expense of which it normally develops. This of course, necessitates variations in virulence in response to the variation in the bacterial species. Moreover, it has been known for a long time that catarrhal diarrhea affects (provoked by the ingestion of undigestible foodstuffs, of green fruits in particular, or by the "froid au ventre" so common in tropical countries) the incidence of certain intestinal diseases—dysentery and cholera among others.

Second, as a result of a more or less marked degree of resistance to the bacteriophage of the invading bacillus. We have seen that in the course of the disease the pathogenic agent defends itself. Such a bacillus in a state of resistance, ingested by a healthy person will develop in spite of the presence of a bacteriophage, particularly if the latter is but slightly active, whereas a non-resistant bacillus is destroyed without a struggle.

In cases of bacillary dysentery, even very severe, but in which the patient improves rapidly, the bacteriophage manifests its presence in a very active manner at the outset, not only for laboratory strains of the bacillus, but for the strain secured from the patient himself, and this takes place at the moment when the symptoms begin to improve. There may be a rapid increase in the virulence of the bacteriophage without a corresponding resistance in the bacterium.

In cases where the disease is prolonged, two cases may be considered:

1. The bacteriophage shows no, or but slight, activity as long as the condition of the patient remains stationary. The improvement occurs when the activity of the bacteriophage manifests itself in an energetic manner, not only for the stock cultures of the bacillus but also for the strain derived from the patient. There has been a delay in the adaptation, then a sudden acquisition of a high virulence. Recovery takes place promptly, for the pathogenic bacterium is not able to develop a resistance.

2. At a given moment of the disease the virulence of the bacteriophage manifests a more or less energetic action on the stock bacilli, but on the contrary, it is inappreciable or but very weak on the strain taken from the patient. Here there has been a delay in the adaptation, since the bacteriophage has gradually acquired virulence for the pathogenic bacillus, but this has allowed sufficient time for the creation of a resistant race of the latter. As a result there is a struggle, and the condition of the patient reveals the fluctuations of the struggle.

This conflict is particularly to be noted in cases of long duration with a relapse. During the latter, especially, the virulence of the bacteriophage shows daily fluctuations. At certain times it may be extreme for the stock culture, although uniformly very weak for the strain of the infection. Recovery begins to take place at the moment when the bacteriophage shows an activity as intense for one strain as for the other.

The disease has a fatal issue in two cases:

1. When the bacteriophage exerts no protective action through a lack of adaptation to the pathogenic bacillus. Here there is no struggle at all, and the bacterium develops freely. In the great majority of such cases non-adaptation is the cause of death, which then occurs quickly.

2. In certain exceptional cases the pathogenic bacterium acquires an almost absolute resistance,—a refractory state. And the bacteriophage, whatever the degree of virulence it acquires, remains ineffective. From this moment, when the bacterium becomes equal to the bacteriophage, the entire body is invaded and death ensues after a greater or less length of time.

COLON BACILLUS INFECTIONS

Sometimes the colon bacillus may become pathogenic and may be encountered as the etiological agent in diverse localized infections, or even in septicemias. It at first appears strange that so common an organism, a normal inhabitant of the intestine, should at a particular time develop pathogenicity. There must be "a something" which differentiates the pathogenic *B. coli* from the banal *B. coli*. It is this which I have tried to determine.

Five specimens of infected urine secured from individuals with pyelonephritis have been examined. In all of these cases not only was the colon bacillus present, but there was a mixed culture of *B. coli* and the bacteriophage, as shown by inoculation of the urine on agar. In one of the cases simple plating of the urine on agar gave a colon culture studded with plaques, in the other four, agar cultures made after a bouillon growth gave the same appearance. The colon bacillus possessed a high resistance, although it was not entirely refractory. Thus the struggle continued in the organism. The ordinary *B. coli* is not pathogenic. The resistant *B. coli* becomes so because of its resistance to the action of the bacteriophage.

The history of a morbid condition is the history of the struggle between the bacteriophage which attacks with its virulence, on one side, and a bacterium susceptible of resistance on the other. Moreover, the struggle can be continued as long as the bacterium secretes products toxic for the infected body, but in the last analysis, it is the issue of this conflict which decides the fate of the individual.

We will have occasion to return to the case of pyelonephritis when we discuss "carriers."

TYPHOID FEVER AND THE PARATYPHOID FEVERS

Several cases of typhoid fever of varied severity have been studied by the same method as that employed in bacillary dysentery. Fourteen of these were in the Pasteur Hospital for treatment, and of these the stools were examined at least once a day throughout the course of the disease and in convalescence. Fourteen more under treatment in other hospitals were followed

with somewhat fewer examinations. In all the charts which follow, the following data is presented;—in the upper portion is the curve showing the temperature; in the lower portion there are three tracings, (1) in dotted line, showing the curve of the virulence of the bacteriophage for *B. coli*, (2) in broken line, showing the virulence of the bacteriophage for an old laboratory strain of *B. typhosus*, a strain which has undergone a great many transfers on laboratory media (this same strain was used in all the cases studied), and (3) in solid line, indicating the curve of virulence

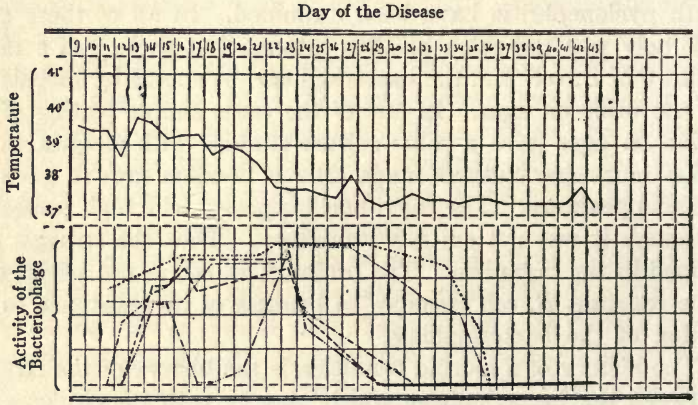

FIG. 6. MARIE MO. . . . . (55 years) CLINICALLY, TYPHOID FEVER

Virulence for { *B. typhosus* - - - - - - -
B. coli . . . . . .
B. paratyphosus A ----------
B. paratyphosus B ----------
B. dysenteriae Shiga . . . . . }

of the bacteriophage for the strain of *B. typhosus* from the patient himself, isolated either by stool culture or by blood culture.

In order to use bacilli as comparable as possible with those found in the body of the patient the strains were transplanted as infrequently as possible. In each case an agar tube was inoculated with a colony taken from the primary culture, and each time that a fresh culture was needed for the preparation of suspensions against which the filtrates containing the bacteriophage from the patient were to be tested, it was always taken from this tube. In this way, the bacteriophage throughout the course

of the disease was tested against a culture as nearly constant as possible, uniform especially from the point of view of the resistance of the bacterium.

For the first three curves only (figures 6, 7, and 8) the organism of the patients had not been isolated (they had fevers which appeared benign) and the curves of the virulence of the bacteriophage against the bacillus of the patient is, of course, lacking. For these three cases the virulence of the bacteriophage against

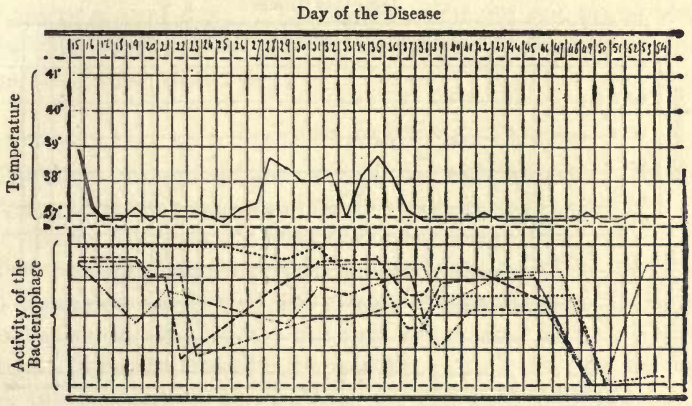

FIG. 7. LOUIS PI. . . . . (17 years) CLINICALLY, TYPHOID FEVER

Virulence for { *B. typhosus* - - - - - - -
B. paratyphosus A ----------
B. coli.....
B. paratyphosus B ----------
B. dysenteriae Shiga .....

a Shiga dysentery strain, and against the paratyphoids A and B are given.

We will select as examples cases of different severity.

1. Mild infections

These were cases of typhoid fever or paratyphoid fever with a mild course. Clinically they were typhoid fever but the blood and stool cultures were negative. The curves for these three cases are given on pages 190, 191 and 192.

1. Marie Mo. . . . (fifty-five years, fig. 6).
2. Louis Pi. . . . (seventeen years, fig. 7).
3. François Jod. . . . (thirty-four years, fig. 8).

In these cases the virulence of the intestinal bacteriophage was determined for *B. coli*, *B. typhosus*, *B. paratyphosus A* and *B*, and *B. dysenteriae* Shiga. It is needless to comment on these observations, since examination of the curves is more instructive than would be an explanation.

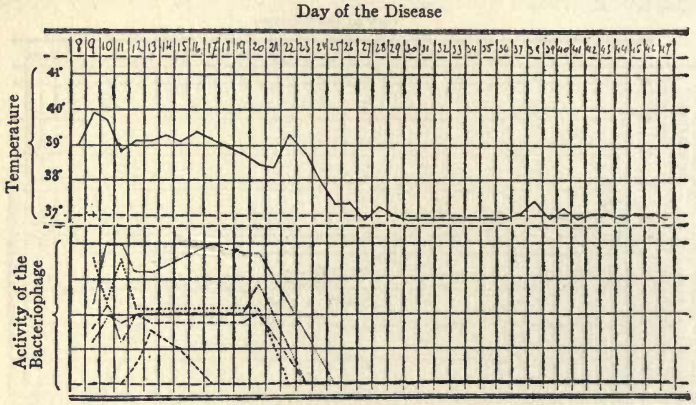

FIG. 8. FRANÇOIS JOD. . . . . (34 years) CLINICALLY, TYPHOID FEVER

Virulence for { *B. typhosus* - - - - - - -
B. coli . . . . . .
B. paratyphosus A ----------
B. paratyphosus B ----------
B. dysenteriae Shiga . . . . . }

What is the causative bacillus in each of these three cases? It is indeed difficult to make a diagnosis by means of the bacteriophage, which as we have seen, but rarely develops a single virulence.

This virulence extends to other bacteria of the same group to a more or less marked degree, and this fact is particularly in evidence when working with the representatives of the colon-typhoid-paratyphoid-dysentery group. It appears, however, in the case of Louis Pi. . . . that the causative bacillus must have been the typhoid bacillus, with Marie Mo. . . . *B. paratyphosus A*, and in François Jod. . . . *B. paratyphosus B*.

It should be noted that in these three cases in which improvement was rapid, the curves representing the virulence of the bacteriophage are comparable. It is also to be noted that in all, the accessory virulences for *B. dysenteriae* Shiga and for *B. coli* are very high, and that the acquisition of virulence for *B. typhosus* and *B. paratyphosus A* and *B* is early and is maintained up to the beginning of convalescence. In the case of Louis Pi. . . . the abrupt deflection in virulence on the twenty-second day preceded a slight relapse which occurred on the twenty-fifth to the thirty-second day. This complication did not prove serious since the virulence of the bacteriophage increased gradually from the twenty-third day.

2. Severe infections

The three cases cited here were serious, with both stool and blood cultures positive. All were infected with *B. typhosus.*

1. Renée Mar. . . . (thirty-two years, fig. 9).

The bacteriophage was from the beginning virulent for *B. coli,* and remained so during the course of the disease, throughout convalescence, and up to the time when the patient was discharged from the hospital completely cured. It may be noted that the acquisition of virulence by the bacteriophage for the bacillus of the infection coincides with the first defervescence. Then this virulence became reduced and the temperature again went up. The infection is definitely overcome at the time when this virulence is again established.

2. Juliette Ou. . . . (thirty-six years, fig. 10).

3. Jeanne Del. . . . (twenty years, fig. 11).

The curves for these two cases are self-explanatory.

3. Typhoid fever with relapse

1. Gilberte Fon. . . . (four years, fig. 12).

On the sixteenth day the disease appeared ended. However, the virulence of the bacteriophage toward the bacillus of the patient disappeared before the end of the crisis and the destruction of the pathogenic bacteria was not complete. Whereupon there was a relapse, very severe, which did not show improvement until the bacteriophage recuperated with a virulence sufficient to control the resistance of the bacterium.

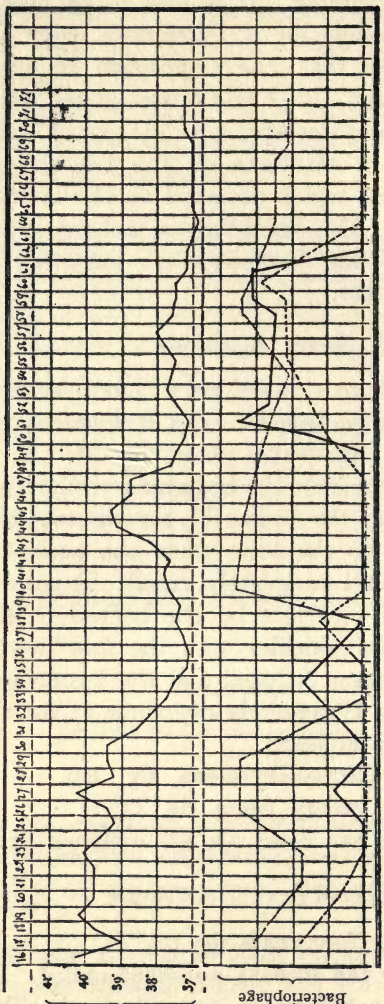

FIG. 9. RENÉE MAR. . . . . (32 years) TYPHOID FEVER (*B. typhosus*)

Virulence for { *B. typhosus* from the patient——
B. typhosus, stock culture - - - - - - -
B. coli. . . . . }

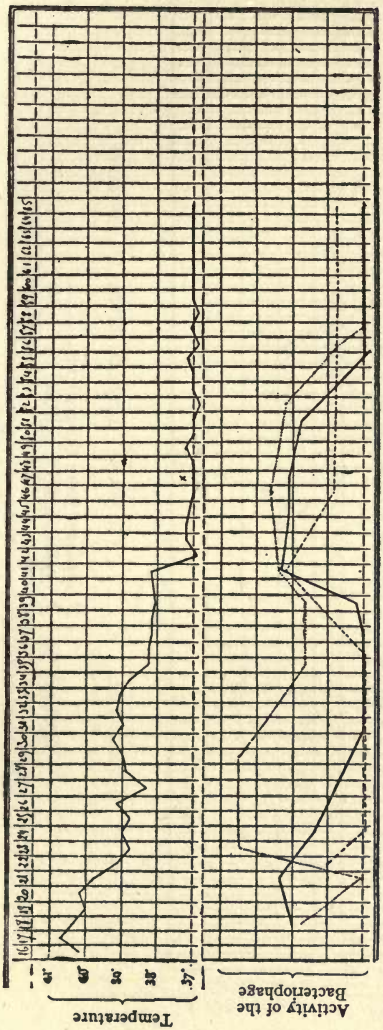

FIG. 10. JULETTE OU. . . . . (36 years) TYPHOID FEVER (*B. typhosus*)

Virulence for { *B. typhosus* from the patient——
B. typhosus, stock culture - - - - - - -
B. coli. . . . . .

"x" Convalescence

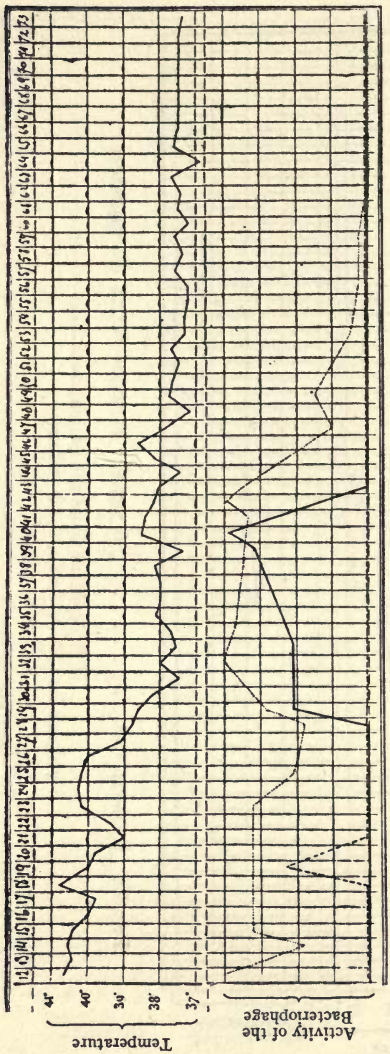

FIG. 11. JEANNE DEL. . . . . (20 years) TYPHOID FEVER (*B. typhosus*)

Virulence for { *B. typhosus* from the patient———
B. typhosus, stock culture - - - - - -
B. coli. . . . . .

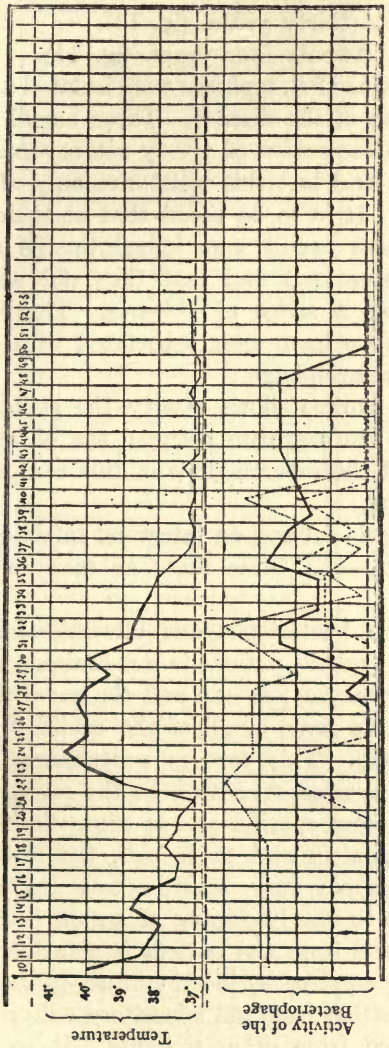

FIG. 12. GILBERTE FON. . . . . (4 years). TYPHOID FEVER (*B. typhosus*)

Virulence for { *B. typhosus* from the patient——— / *B. typhosus*, stock strain - - - - - - / *B. coli*. . . . . }

———Condition serious

4. Typhoid fever of extreme severity

1. Andrée Dess. . . . (thirty years, fig. 13).
2. Jeanne Cot. . . . (twenty-four years, fig. 14).

In these two cases strains of *B. typhosus* were isolated at different times during the course of the disease. These bacilli presented a marked resistance to the action of a very active strain of anti-typhoid bacteriophage and lost this resistance only after about ten transfers on agar. It is to be noted that at their isolation from the organism these bacilli were inagglutinable (this fact has frequently been observed) and that they did not become agglutinable until after a series of cultures. This transitory inagglutinability is, as we have seen, associated with resistance to the action of the bacteriophage.

Examination of the curves shows clearly the struggle which was carried on within the organism between the bacterium and the bacteriophage, and the repercussions of this campaign upon the state of the patient.

We find then, in typhoid fever,—an intestinal infection complicated by a septicemia—the same facts as seen in bacillary dysentery.

The virulence of the bacteriophagous ultramicrobe isolated from the stools of the typhoid patient is not limited, in general, to a single pathogenic bacillus; at one and the same time it extends, in some degree, to some or all of the bacilli of the colon-typhoid-dysentery group. This fact is particularly noted in mild cases or those of average severity. In the severe cases the bactericidal action is more specific and is often limited to the specific pathogenic organism and to *B. coli*, the latter always being attacked. In certain very severe cases the specificity becomes such that up to the beginning of actual improvement only the bacillus isolated from the patient is attacked, whether it has been secured by stool or by blood culture, to the exclusion of other bacilli, taken either from old laboratory cultures or from strains recently isolated from other patients. It seems, then, that in the course of their struggle each of the two organisms present,—bacteriophage and bacterium—acquires an individual personality, which differentiates them from other organisms of

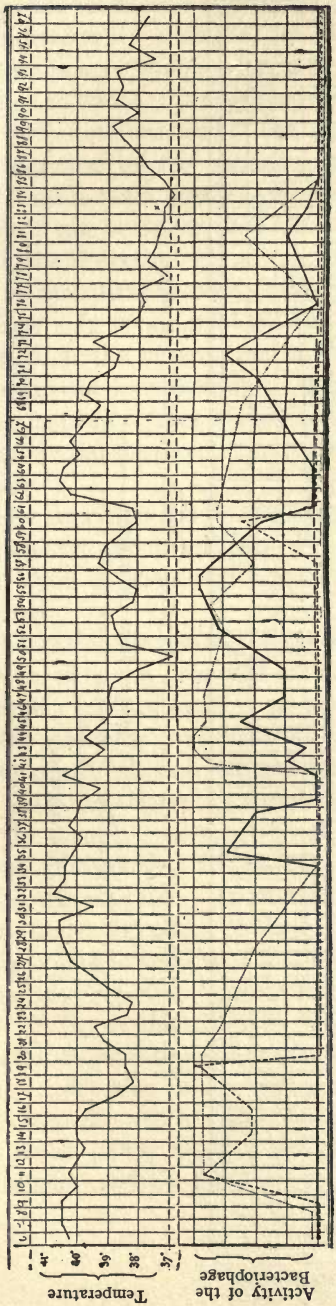

FIG. 13. ANDRÉE DESS. . . . . (30 years) TYPHOID FEVER (*B. typhosus*)

Virulence for { *B. typhosus* from the patient——
B. typhosus, stock strain - - - - - - -
B. coli. . . . . }

"x" Convalescence

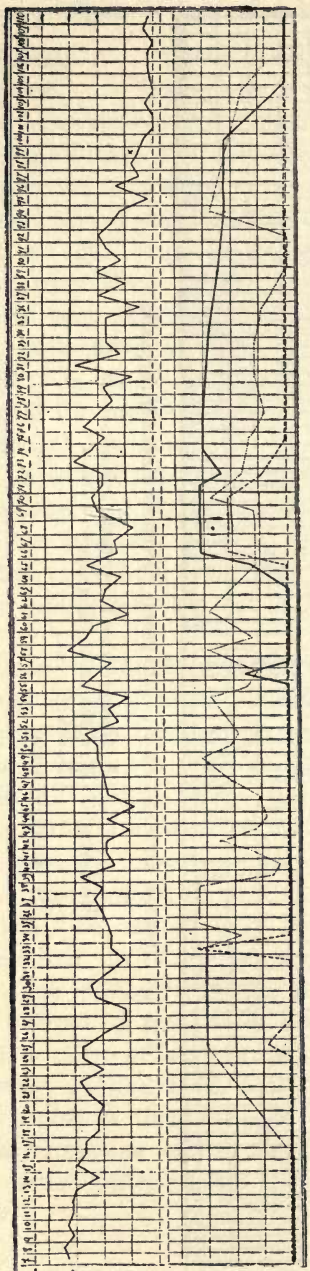

FIG. 14. JEANNE COT. . . . . (24 years) TYPHOID FEVER (*B. typhosus*)

Virulence for { *B. typhosus* from the patient——
B. typhosus, stock strain - - - - - - -
B. coli. . . . . .

"x" Convalescence

the same species rendered banal as a result of cultivation. However, this individuality is effaced by cultivation, both in the case of the bacteriophage, which, after a few passages at the expense of the bacillus to which it is sensitive may develop activity toward any strain of *B. typhosus*, and of the typhoid bacillus which is then able to be attacked by any strain of antityphoid bacteriophage.

Typhoid fever is not a purely intestinal infection as is dysentery. In the latter it can be understood how, when all of the pathogenic bacteria of the intestine or of the mucosa, that is, those in proximity to the ultramicrobes, have been destroyed the disease ends *ipso facto*. In typhoid fever there is in addition a septicemia and even though the destruction of the bacilli contained in the intestinal contents is sufficient to delay the appearance of the disease or to restrain it from the beginning, it may not be adequate to overcome the infection once the pathogenic bacilli have invaded the organism.

We will see later that the protective action of the bacteriophage is not limited to the intestine. The intervention of the bacteriophage, even in the same organism, may manifest itself in different ways.

In the chapter dealing with the properties of the bacteriophage we have seen that the products which it secretes are possessed of an extremely high opsonizing power. A culture of an antityphoid bacteriophage is precipitated by the addition of four volumes of 96 per cent alcohol. The precipitate is allowed to remain in contact with the alcohol for forty-eight hours, a time adequate to ensure the complete destruction of all of the bacteriophagous germs. One centigram of this moist precipitate is dissolved in ten cubic centimeters of saline. In determining the opsonic index, it will be seen that under the action of the lysin the leucocytes become so loaded with typhoid bacilli that it is impossible to count the number of organisms ingested. The opsonic index is certainly higher than fifty. It is possible that the lysin secreted in the intestine as soon as the bacteriophage has acquired a virulence to dissolve the typhoid bacilli may be resorbed and pass into the circulation, and thus assure the destruction of the bacilli by phagocytosis.

On the other hand, the bacteriophagous ultramicrobe itself does not remain strictly localized in the intestine; at times it

passes into the circulation. This has not been demonstrated in man for it is not practicable to carry out on man the repeated blood examinations which such a study requires.[4] However, in paratyphoid fever in the rat induced by the ingestion of a very virulent strain of *B. typhi murium* a transitory appearance of the ultramicrobe in the blood has been demonstrated by cardiac puncture made between the fourth and sixth days after the ingestion of the infectious material. All of the rats in which this phenomenon occurred were protected. At this time a bacteriophage active for the pathogenic bacillus was present in the intestine and the rats resisted infection.

In the third place we will see experimentally that the dissolved products found in the cultures of the bacteriophage provoke, after an incubation period, the development of an "organic immunity" so potent that it borders on a refractory state. These dissolved products likewise form in the intestine of the patient, and even within the body, since it is possible for the bacteriophage to pass into the circulation in a septicemia.

Twenty-eight more non-fatal cases of typhoid fever were studied in order to determine the influence of the bacteriophage on the course of the disease. Three fatal cases were observed. In these three cases, at no period of the disease could the presence of a bacteriophage be demonstrated active for *B. typhosus*, either for a stock strain or for the bacillus from the patient. Furthermore, examination of the strains from the intestinal contents from five individuals who had died of typhoid failed to show any activity for the typhoid bacillus. But the bacteriophage was not entirely absent, since in six of these eight cases a bacteriophage of moderate activity for the colon bacillus was found. This bacteriophage did not, however, show any activity for the pathogenic organisms. Death in typhoid fever results, usually, because of a failure of the bacteriophage to adapt itself for the bacteriophagy of the invading bacillus.

May death occur because of the acquisition of a resistant condition by the typhoid bacillus, which protects it from the action

[4] Beckerich and Hauduroy have very recently found it in blood cultures. It is essential to examine them systematically for the bacteriophage; the negative cultures in particular.

of the bacteriophage, as we have seen in the case of dysentery? There has been no opportunity to establish this up to the present but it is the more probable, since, *in vitro* as *in vivo*, the tendency toward resistance is certainly more marked for the typhoid bacillus than for *B. dysenteriae*. In any case, this cause of death is certainly the exception, even in typhoid. It must necessarily accompany a septicemia when it occurs.

In typhoid, as in dysentery, the investigation of the virulence of the bacteriophage is of prognostic significance. It is sufficient to verify simultaneously the virulence of the intestinal bacteriophage of the patient toward *B. coli*, toward the pathogenic bacillus taken from the patient, and toward a stock culture of *B. typhosus*. A comparison of these three results furnishes the information desired. The detection of resistance in the pathogenic bacterium would indicate a poor prognosis, and that in proportion as the resistance is the more pronounced. The establishment of a refractory state in the bacterium, resulting in the formation of a mixed culture in the intestine accompanying a septicemia, implies a fatal outcome with a quick maturity.

To summarize: in all of the cases of typhoid fever studied, whatever may have been their severity, the appearance in the bacteriophagous ultramicrobe of virulence for the pathogenic bacillus has been preceded by an increase in virulence for *B. coli*, which has always begun in the course of the second week and has rapidly attained great intensity. This activity is maintained during the entire course of the infection and appreciably decreases only during convalescence, sometimes even later. On the contrary, the development of virulence for the pathogenic bacillus has varied according to the severity of the disease. In cases that were mild or of average severity the activity of the bacteriophage for this bacillus appears before the end of the second week and disappears toward the end of convalescence. The activity for *B. coli* and for *B. typhosus* is there parallel. In the severe cases the activity for the typhoid bacillus only commences to manifest itself in an energetic manner towards the beginning of definite improvement. It persists for a greater or less length of time, in some cases up to the middle of the period of convalescence.

In the forms with relapse and recrudescence the struggle is complicated by the fact of the acquisition of a resistance by the

bacteria, and it is only toward the decline of this relapse or of the recrudescence that the virulence of the bacteriophage is sufficient to definitely control the resistance of the bacterium. Here, the activity of the bacteriophage is maintained up to complete recovery, that is to say, up to the moment when, because of a total destruction of the pathogenic bacteria, the ultramicrobe is no longer able to develop at their expense.

In all cases, the condition of the patient faithfully registers the vicissitudes of the struggle taking place within the body between the bacteriophage and the invading bacterium.

AVIAN TYPHOSIS

The disease

Avian typhosis is a disease affecting principally the Gallinaceae. Despite its frequency it for a long time remained undetected, confounded with chicken cholera. This last disease is, in reality, very rare. In 1919, in investigating epizootics for the purpose of testing on domestic animals, which allow of experimentation, the conclusions reached as to the rôle of the bacteriophage in human dysentery and typhoid, an extended focus of "chicken cholera" was found in the Department of the Aube. In the first examinations the error which had been made became apparent; it was the disease known in the United States as "fowl typhoid," whose existence in France had up to that time been unrecognized. Shortly after this numerous foci throughout the surrounding territory were discovered.

Fowl typhoid, which will here be called fowl typhosis, is a very interesting disease. Its study is complicated by the existence of several "paratyphoses" which resemble still more the human typhoid. The pathogenic agent, *B. gallinarum* Klein, studied by Moore under the name of *B. sanguinarium*, presents, with the exception of motility, all of the characteristics of the bacillus of Eberth (*B. typhosus*). It is even agglutinated to titre by an anti-typhoid serum. Aside from this type bacillus there are often found, in the same foci, bacilli presenting different agglutinative and biochemical reactions. The clinical type of the infection which they provoke does not differ from that caused by the

typhoid type. These differing species of bacteria have up to the present been studied only by American workers; Ph. Hadley among others, who describes *B. pullorum A*, *B. pullorum B*, *B. jeffersonii*, *B. rettgerei*, and *B. pfaffi*. A discussion of the distinctive characters of these different bacilli will not be presented here since it would not be germane to the study with which we are concerned.[5] It is sufficient to know that in France in the epizootic of 1919 the most frequent pathogenic agent was of the *B. gallinarum* type (found in 57 of 73 examinations). Along with *B. gallinarum* other forms have been found:—*B. pullorum A* (once), *B. pullorum B* (6 times), *B. jeffersonii* (4 times), and *B. pfaffi* (4 times). In a single focus, of which the centre was found in the village of Trainel (Aube), a paratyphosis infection occurred due solely to *B. pfaffi* without admixture with bacilli of the true typhosis type.

The clinical picture hardly varies whatever may be the causative bacillus. A typical observation follows.

On the evening of May 24 the chicken appeared perfectly well. On the morning of May 25 it remained apathetically on the ground of the poultry-yard and took no measures for its defense. The next day, toward noon, it appeared somnolent, the plumage rough, the eyes half-closed, the crest slightly violet colored. It did not eat or drink, and remained humped up "in a ball." The inspirations were deep, twenty-five per minute. There was a greenish yellow diarrhea with portions definitely yellow. The condition became worse in the afternoon. It fell on its side at about 8 o'clock and died a few minutes later. The necropsy showed the crest to be violet in color, with spots of the same nature over the skin. The liver was voluminous, congested, and presented foci of degeneration. There was a pericarditis.

By direct microscopic examination the blood at first appeared negative, but a very careful search revealed three bacilli in a whole smear. The blood and tissues when cultured gave a pure growth of *B. gallinarum*, and this organism was also found, very abundantly, in the intestinal contents.

[5] Readers who are interested in the subject will find much useful information in the contribution by Ph. Hadley *"The Colon-Typhoid Intermediates as Causative Agents of Diseases in Birds."* Bulletin No. 174, Rhode Island Agric. Exper. Sta., 1918.

Sometimes death occurs more rapidly still, in certain cases in a striking manner. Epizootics of avian typhosis have a high mortality. In 1919 foci existed throughout the extent of France. In general, the epizootic begins quickly; within the space of three or four weeks a half, three-quarters, sometimes more, of the fowls on a farm succumb. Then the disease assumes a sporadic character, only an occasional animal dying during the course of a year. The disease may disappear for a few months and then reappear. The annual mortality amounts to forty to seventy per cent of the population of the infected poultry-yards. Young adults are the most susceptible, then the old animals; the chicks are in general spared.

Epizootics of typhosis extend rapidly over large areas; certain Departments were contaminated throughout in 1919. The establishment of a new focus begins by the importation of the organism from an infected region, either through the agency of a flock of sheep or herd of cattle, or by horsemen (this last mode of dissemination was particularly frequent during the war; this explains the extension of the disease during the years 1917 and 1918). The disease rages for a few days on a farm, passes to a neighboring farm, and then extends rapidly into the surrounding villages.

The pathogenic bacillus remains alive and virulent during several months in the regions where the infection has been epidemic. In several tests it has been shown that an isolated infected chicken-yard, cleaned and left unoccupied for six to eight months, still contains virulent germs, for, when repopulated with chickens from a region free of the disease, the infection breaks out again within a few days among the new occupants.

Avian typhosis being a disease in general but little known, I have thought it useful to consider it in some detail, since it will allow us the better to understand the facts now to be presented.

The rôle of the bacteriophage in the course of the disease

Because of the exceptional severity of the infection in avian typhosis it has been possible to follow only four cases which recovered. In all, the picture has been identical. In the morning the infected chicken remains on the ground, "balled up,"

the feathers roughened, and with the characteristic diarrhea. The appearance is the same as in the fowls which succumb. At this stage of the infection examination of the feces gives results such as:

B. gallinarum, present in abundance.

Intestinal bacteriophage, virulent for *B. coli* + (in 2 cases) or ++ (in 2 cases); for *B. gallinarum* 0 (in the four cases). The blood culture was positive in the two cases in which it was done; the blood for culture being taken aseptically by puncture of the crest.

During the course of the day the condition remains the same as that shown by animals which die. This state is prolonged and the next morning the chicken still appears the same. Examination of the feces at this time shows:

B. gallinarum present in three cases, absent in one.

Intestinal bacteriophage virulent for *B. coli* +++ (4 cases); for *B. gallinarum* + (in 3 cases) +++ (in 1 case). Towards noon, in one case, in the course of the afternoon in the three others, blood cultures were negative. In three cases a bacteriophage active for *B. gallinarum* was found in the blood. The blood which was ultrasterile was that of the chicken whose condition was the best at this time and which had shown no pathogenic bacilli in the intestinal tract in the morning. The presence of the bacteriophage in the blood is extremely transitory.

On the morning of the third day the animals appeared normal, they drank a great deal, ate some grain, and the diarrhea was less profuse. Examination of the feces showed:

B. gallinarum absent in the four cases.

Intestinal bacteriophage active for *B. coli* +++ (4 cases), for *B. gallinarum* +++ (3 cases) ++++ (1 case). Blood cultures were negative: no bacilli, no ultramicrobes.

On the fourth day the animals were practically normal.

In the four chickens which recovered the bacteriophage remained active for *B. gallinarum* for a very long time. After three months it showed the same degree of activity as at the time of recovery. In one of them, in which it has been possible to make an examination after five months, it was still as active as at first. We will see, from experimental observations that this

persistence of virulence depends solely upon the fact that the pathogenic bacillus, distributed in profusion in the exterior environment, is frequently ingested by the animal and this maintains the virulence of the intestinal bacteriophage since it is able to grow at its expense.

The feces of about one hundred chickens which had died of avian typhosis were examined. In no case was there a bacteriophage active for *B. gallinarum* or for any of the bacillary agents of the paratyphoses. Nevertheless the bacteriophage had been present for it could be disclosed (91 times in 97 examinations) because of the activity shown for one or several species of the colon-typhoid-dysentery group. One sees clearly, then, that the lack of defense is not due to the absence of the bacteriophage, but solely to the fact that the intestinal bacteriophage remained passive because it failed to acquire a virulence for the pathogenic bacillus.

To summarize: as in dysentery and in typhoid fever in human beings, the acquisition of virulence by the intestinal bacteriophage for the pathogenic bacterium is the *sine qua non* of recovery.

Rôle of the bacteriophage in the course of the epizootic

Because of the dissemination and extent of the disease it was possible to study the rôle of the bacteriophage in the course of the epizootic as well as in the course of the disease in the individual infected animal.

Let us consider first a fact bearing on the territory involved in the epizootic. During the last three years eighty-one examinations have been made upon the feces of barn-yard animals, not only in France but also in Indo-China, in regions where avian typhosis had not occurred in epidemic form among the fowls for several years. In each of these examinations a bacteriophage active for one or several of the bacilli of the colon-typhoid-dysentery group was isolated, but in no instance has the bacteriophage shown any detectable activity for *B. gallinarum.*

In contaminated regions the situation is quite different. As an example, observations made on a farm located at Pougy-sur-Aube may be cited, where the disease was followed very closely. The disease appeared in 1917 in July. Within the period of a

month fifty-one of the ninety-eight fowls died; then the epizootic disappeared. In May, 1918 it reappeared in less violent form. Twenty-five of one hundred and four fowls died in the period from May to September, and it again disappeared. In 1919 it broke out again early in April. On the 21st of May, twenty-one of eighty had died. At this time I began my observations.

On May 21, specimens of the excrement of thirty of the fifty-nine survivors were taken. Examination, made later in the laboratory, showed in twenty-six a bacteriophage of weak or moderate activity for *B. gallinarum* (23 were +, 3 were ++), in four it was absent. On May 22, two chickens contracted the disease. The strains taken the day before were numbered and examination showed that an active bacteriophage had not been found in these two animals. On May 23 one of the two chickens affected the day before died. On May 24, a third chicken, sick in the morning, died in the following night. Its excrement, collected on May 22, did not contain a bacteriophage active for *B. gallinarum.* On the morning of May 24 the chicken which had been taken sick on May 22 and which had resisted showed in its intestinal contents a bacteriophage of extreme activity (++++) toward the pathogenic bacillus. On May 26 the fourth chicken, one of those whose feces had not showed an active bacteriophage when examined on May 22, was affected. It resisted, and on May 28 its symptoms had disappeared. The disease disappeared suddenly and during the next three months no new cases developed.

On May 30 the feces of thirty chickens were examined and the following results were obtained:

Virulence for *B. gallinarum;* in five ++++, in twenty-one +++, in four ++.

We see, then, on May 22, four animals among thirty in which the intestinal bacteriophage lacked activity for the pathogenic bacillus. These four animals contracted the disease during the four following days. In the twenty-six specimens collected on May 22 and showing positive results, the bacteriophage showed a relatively weak virulence. Nine days later this activity was very much greater, that is, at the time when the epizootic ceased. What, then, took place in this interval? The bird which became sick on May 22 and which resisted showed in its feces, when ex-

amined on May 24, a bacteriophage endowed with a considerable activity for the pathogenic agent.

Here is a second example of the same general nature, giving the results secured on farm M. . . . at Véricourt (Aube). The epizootic first appeared among the flock of twenty-five chickens in May, 1919. The first animal died on May 18. On the next day twelve specimens of excreta were collected at random. Three only contained a bacteriophage, and that of feeble activity, for *B. gallinarum*. From May 19 to 26 twelve birds contracted the disease and of these eleven died. One, which became sick on May 23, showed on May 25 a strongly active bacteriophage (+++ for *B. gallinarum*) and recovered. The epidemic stopped abruptly. On May 27 twelve specimens were taken at random. In all a bacteriophage active for *B. gallinarum* was found (in 1 ++++, in 9 +++, in 2 ++).

A third example may be mentioned, in which the infection was a paratyphosis.[6] On October 15 strains of *B. pfaffi* were isolated from two specimens of blood, taken from animals which had died in a chicken-yard where for about a month there had been an infection presenting the characters of typhosis. From specimens of the feces taken from two healthy animals living in the same yard two strains of bacteriophage were isolated, one showing a low virulence (+) for *B. pfaffi*, the other showing no activity for this bacillus. Towards the end of the month three chickens became sick, recovered after an interval of two or three days, and then the epizootic ceased. Six specimens of feces examined at this time all showed a bacteriophage of high virulence (+++) for *B. pfaffi*. Against *B. gallinarum* four were inactive and two showed a weak virulence (+).

B. pfaffi was therefore the cause, for when the epizootic broke out three months later the eighty chickens which had survived received a subcutaneous injection of 0.5 cc. of a culture of the anti-pfaffi bacteriophage and the epidemic stopped abruptly and permanently from the time of the injection. We will see later that this abrupt cessation is the rule following immunization by means of a culture of the bacteriophage.

[6] These experiments were carried out with the assistance of M. Micheau, D. V. M. at Trainel (Aube).

These facts can be explained in only one way. A weak or moderate activity of the intestinal bacteriophage for the pathogenic bacterium is sufficient to render the animal resistant to infection. The pathogenic bacteria which are able to penetrate into the intestine are destroyed before they can multiply. But it is not the same once the disease has appeared and the organism is invaded. The animal recovers, and this is very rare in typhosis, only because of a rapid adaptation of the bacteriophage and the acquisition of a high virulence which leads to an intensive destruction. This bacteriophage with exalted virulence is distributed broadcast with the excreta of the recovered or convalescent animals, and continues, indeed, during several months after recovery. This bacteriophage is necessarily ingested by the other animals of the barn-yard which become, in fact, "infected" by an extremely active bacteriophage and by this means acquire a complete protection against the disease, in spite of the presence of the pathogenic organism in the environment, and in spite of its frequent ingestion, an ingestion which serves to maintain the virulence of the bacteriophage.

These hypotheses are not simply idle speculation, for the interpretation given to these observed facts is confirmed by experiments which provide in a controlled manner the natural conditions of the epizootic. Furthermore, it will be seen that the rôle of defense assigned to the bacteriophage is confirmed by the immunization of several thousand animals by the administration of cultures of an active bacteriophage.

Before discussing these control experiments I ought to mention that, thanks to the kindness of the veterinarians of different regions invaded by typhosis, I have been able to procure numerous specimens of blood and excreta taken from sick chickens, from chickens which had died or from those which had recovered, derived from eleven different foci scattered throughout all France. This allows me to generalize from the facts that I have personally observed.

Control experiments

The control experiments have been conducted in Paris, that is to say, entirely outside of the epizootic area.

Six chickens, procured from a region free of infection, were placed under observation. Their excreta were examined daily for ten days for the purpose of establishing the complete absence of a bacteriophage active for *B. gallinarum.*

Chicken no. 1 then received, *per os,* 1 cc. of a culture of a strain of bacteriophage very active for *B. gallinarum* (++++).

Chicken no. 2 received 0.5 cc. of the same culture by subcutaneous injection.

The next day examination of the feces of these two animals showed the presence of a bacteriophage strongly virulent for *B. gallinarum.* Therefore, the bacteriophage passed into the intestine, whether ingested or injected. This same fact has since been verified with man and with different animals.

Chicken no. 1 next received *per os* daily for twenty-five days, 2 cc. of a bouillon culture of *B. gallinarum.* The active bacteriophage persisted in the intestine with its primary virulence (++++) and maintained itself up to nine days after the last dose of the pathogenic organism.

Chicken no. 2, which had received nothing after the inoculation of the active bacteriophage ceased to show an active strain for *B. gallinarum* within three days after the injection. In other words, chicken no. 1, subjected to repeated reinfections, retained an intestinal bacteriophage active for *B. gallinarum* for thirty-four days, while chicken no. 2, not infected, for only three days.

It follows that the intestinal bacteriophage remains active only if it is able to develop in the intestine at the expense of this bacterium, but in such a case it remains active just so long as this condition is fulfilled. Inversely, the presence in the intestine of a bacteriophage possessing virulence for a given bacterium indicates that this bacterium was a short time previously in the intestine.

In the course of the preceding experiment chickens nos. 3 and 4 were placed in contact with chicken no. 1. They all ate and drank from the same containers, the more so since they were changed about in the pens in such a manner as to simulate conditions of life analogous to those of the chicken-yard. Two days after the first contact, in the case of chicken no. 3, three days after with chicken no. 4, their excreta contained a bacteriophage very

virulent for *B. gallinarum* (++++). From this time on they each received each day for twenty-one days, 2 cc. of a bouillon culture of *B. gallinarum*. At no time did they appear sick. The intestinal bacteriophage remained active for the bacillus throughout the entire period of the administration of the pathogenic bacillus, and even longer—seven days in no. 4 and ten days in no. 3. The intestinal bacteriophage did not then disappear, for as in the case of chickens nos. 1 and 2, it remained active for one or several members of the colon-typhoid-dysentery group. But the virulence for *B. gallinarum* did not persist when the ingestion of cultures of this last bacillus was stopped. The experiment with chickens nos. 3 and 4 shows clearly that the bacteriophagous ultramicrobe is infectious in exactly the same sense as is the pathogenic bacillus itself, since these birds were "contaminated" by contact with chicken no. 1.

Chickens nos. 5 and 6, which had not been in contact with the other chickens, and which on repeated examinations were shown to be free of a bacteriophage active for *B. gallinarum*, each received *per os*, on some bread, a single dose of 2 cc. of a bouillon culture of *B. gallinarum*. Three days after the infecting meal diarrhea appeared and they died two and three days later, after having shown all of the symptoms of the natural disease. Necropsy showed the presence of the same lesions. Cultures of the blood gave pure cultures of the pathogenic bacillus, which was likewise found in abundance in the intestinal contents.

Chickens nos. 1, 3, and 4, which had resisted repeated ingestions of *B. gallinarum* culture without showing the least inconvenience, were therefore immunized; the first as a result of the ingestion of a bacteriophage active for the pathogenic bacterium, the two others by simple association with the first.

About one month after the virulence of the bacteriophage for *B. gallinarum* had disappeared in chickens nos. 1, 2, 3 and 4 each of them was given on each of three days 2 cc. of a culture of the bacillus. In all the intestinal bacteriophage showed a new virulence for the pathogenic organism. None of them showed the slightest trouble.

In all of these experiments the infections have been made with bouillon cultures of *B. gallinarum* prepared directly from the

blood of chickens dead of spontaneous natural infection. This is essential because of the loss in virulence of this organism which takes place under artificial cultivation.

With chickens nos. 5 and 6 the ingestion of the pathogenic bacillus caused a fatal attack of typhosis. The intestinal bacteriophage at no time manifested an activity for the causative organism. In chickens nos. 1, 2, 3, and 4, on the contrary, the ingestion of the same culture caused no disturbance and their intestinal bacteriophage which for about a month had showed no activity for the bacillus, rapidly recuperated its first activity. It had, therefore, not disappeared from the intestine, although its activity was no longer evident, but when it found itself again in contact in the intestine with the pathogenic organism it rapidly regained its potency.

This "latent virulence" may be maintained for a very long time. In this connection I may recall the fact cited of a strain of bacteriophage still possessing after three years and more than 1000 passages *in vitro*, always with the Shiga bacillus, the power to attack *B. coli* and *B. typhosus*. It showed a weak power, but was capable of rapid augmentation by transfers at the expense of these organisms. This is exactly what this experiment shows us to take place *in vivo* in the chicken.

Can a chicken contract typhosis in spite of the presence of an active bacteriophage in the intestine? It certainly can. As we have seen in many experiments the bacterium may develop a resistance to the action of the bacteriophage and this resistance is one of the factors comprising the virulence of the bacterium. We have then, on the one hand, the bacterium, which when introduced into the organism may acquire a resistance to the action of the bacteriophage ranging from zero to absolute resistance, and on the other hand, the bacteriophage, which at the same time may possess a virulence running from zero to extreme activity. Infection occurs, or does not occur, according to whether the algebraic sum of virulence + resistance is in favor of the one or the other of the two organisms present. Once the disease has manifested itself, the virulence of the one and the resistance of the other become increased or attenuated according to the conditions of the moment and the aptitudes previously acquired

favoring the one or the other of the two germs. The sequence in which the events of the struggle unfold determine the issue.

Conclusions

The observations made in natural disease and the experiments which confirm the deductions which these observations suggest, show that the bacteriophagous ultramicrobe is always present in the intestine of the chicken, whether it is healthy or sick, whether it lives in a locality free of infection or in an epizootic zone.

Against a definite bacterium, *B. gallinarum* in so far as avian typhosis is concerned, the intestinal bacteriophage may be virulent or avirulent, and in the first case its virulence may be exercised according to a scale which passes from the smallest degree capable of detection to one of extreme activity.

Virulence of the bacteriophagous ultramicrobe for *B. gallinarum* is only observed in an infected locality. The absence of such a virulence is equally the rule with animals which are about to die and with those which have died.

In a contaminated area animals which harbor in their intestine a bacteriophage endowed with sufficient virulence for the pathogenic bacterium are by this very fact protected against the disease, and they remain so, provided the actual or latent virulence is maintained at a level sufficiently high to effect a rapid destruction of the pathogenic bacilli ingested.

The ingestion of pathogenic bacilli at sufficiently frequent intervals constitutes the principal factor in maintaining the virulence for the given bacterium. Among the factors which contribute to diminishing the virulence or causing the virulence of the bacteriophage for a pathogenic bacterium to disappear, I would place as most significant the introduction into the organism of bacteria endowed with resistance to the action of the bacteriophage. We have clearly seen this fact in the course of the experimental study of the phenomenon of the resistance of bacteria. Another possible factor, influencing the activity of the bacteriophage is the reaction of the medium in the intestine, which may vary according to the accidental conditions of the moment, the type of food, etc. The importance of the reaction of the medium has already been shown for lysis *in vitro*.

A bacteriophage which has lost its virulence for the pathogenic bacterium lacks the power to exercise it because of the absence of this bacterium, but it possesses nevertheless, a latent virulence. When placed again after a greater or less length of time in the presence of this bacterium it regains its original virulence.

The fact of the habitual virulence of the intestinal bacteriophage for *B. gallinarum* in the infected regions indicates the frequency of the ingestion of these bacilli, and consequently the excessive contamination of the environment by the pathogenic organism.

In contaminated regions the animal in which the intestinal bacteriophage does not enjoy any activity for *B. gallinarum*, quickly contracts the disease. It may resist and recover, but this is the exception, occurring only when the intestinal bacteriophage quickly acquires a virulence for the infecting bacillus. In the contrary, and usual, case the animal succumbs.

In a chicken which recovers, the intestinal bacteriophage acquires a considerable virulence against the pathogenic bacterium and maintains this for a very long time; in fact, as long as the exterior environment remains infected. This persistence of virulence is maintained by the frequent ingestion of pathogenic organisms, which allow the bacteriophage to multiply at the expense of the particular organism. The resistant animal disseminates in its excreta the bacteriophage of enhanced virulence; the animals which associate with it become "contaminated" and by this fact they enter the same class of resistant animals as those which have recovered. Recovery of one animal in a barn-yard often marks the end of an epizootic, or its arrest for a few months.

The study of an epidemic of avian typhosis shows, in a word, that the history of the contagion reflects, in the last analysis, the story of the struggle between the two agents—the pathogenic bacterium and the bacteriophagous ultramicrobe—and since this last is transmissible from individual to individual the immunity is contagious in the same sense as the disease itself. The beginning of an epizootic is marked by a diffusion of the bacteria, the end by a diffusion of a bacteriophage virulent for these bacteria. We will encounter the same facts in another disease; in hemorrhagic septicemia in the buffalo.

HEMORRHAGIC SEPTICEMIA OF THE BUFFALO (BARBONE)[7]

Barbone, the disease

Contrary to the diseases discussed up to this point, barbone does not present intestinal symptoms; it is of the hemorrhagic septicemia type. The pathogenic organism is a Pasteurella. Cultures of the organism in beef bouillon maintain their virulence for a considerable time—at least eighteen months. The inoculation of a buffalo or of a cow with 0.0002 cc. of a virulent culture kills the animal in between thirty-six and forty hours with all the symptoms of the spontaneously acquired disease. At necropsy identical lesions are found and the pathogenic bacterium swarms in the blood and in the organs.[8]

The buffalo is par excellence the beast of burden in the cultivation of rice-fields; it replaces the ox in all southern Asia and in the islands of the Sunda Straits. It is utilized in certain regions of Italy, in Egypt, in Hungary, and in the Balkans. Wherever the buffalo lives there also will be found barbone, the most terrible, without doubt, of all the contagious diseases. The reports indicate a mortality of from 70 to 95 per cent. I was present during an epizootic which raged in June, 1920, in the Province of Bac Lieu (Cochin-China) where among the thirty thousand buffaloes of the region ten thousand died, and I did not have an opportunity to observe a single animal which recovered. Recovery may occur, but it is certainly rare, and the mortality in Cochin-China is certainly above 99 per cent of the animals affected.

The average duration of the evolution of the disease is but eighteen to twenty-four hours; rarely thirty-six. Death sometimes takes place without precursory symptoms. An animal yoked to a plow stops, remains motionless for a few moments with an

[7] The experiments on barbone have been performed in collaboration with G. Le Louet, Chief of the Veterinary Service in Cochin-China.

[8] In two different attempts I have proved that diluted blood or macerations of organs (liver and lung) taken from animals dead of spontaneous infection, filtered through a Chamberland filter (L_2) and inoculated in large amounts into the buffalo or into cattle do not cause the slightest disease symptoms.

haggard aspect and then falls as though struck by lightning. In typical cases, which can be reproduced in a perfect manner in experimental infection, the animal appears dejected, the eyes fixed, the head lowered. The temperature rapidly mounts to 41.5 to 42.5°C., the respiration, at first accelerated, becomes slowed and then dyspneic, the inspirations less and less frequent. The animal shows meteorism; it lies flat on the ground in complete lateral decubitus usually a short time before death which is preceded by cramps and at times convulsions.

Often tumefaction is to be observed, appearing usually in the region of the throat and extending back to the shoulder. The engorgement is produced by a gelatinous exudate of a yellow color within the connective tissue. At times the tumefaction appears in another part of the body, or it may be entirely lacking. This tumefaction, as shown in experimental infection, marks the portal of entrance of the pathogenic bacteria. Infection usually occurs by way of the digestive tract and the virus most frequently penetrates the tissues through some portion of the nasopharynx. A tumefaction on another part of the body—thigh, abdomen, rump—indicates a reinfection by the penetration of the virus through an excoriation. Examination of cadavers shows that the absence of tumefaction indicates an infection by way of the stomach and intestine.

Bovines and the buffalo are equally susceptible, as was noted a long time ago by Piot in Egypt. The statistics of Indo-China indicate, it is true, that the mortality from barbone is but slight for cattle, but this is solely due to the fact that these animals are present in but small numbers in the regions where barbone rages; regions which are extremely humid and admirably adapted to the buffalo, a semi-aquatic animal. The rare cattle found sometimes in such regions contract the disease and die like the buffalo, after having presented identical symptoms.

The effect of low places and swamps on the contagion has been from time immemorial recognized by the natives. When it is possible, as soon as a case of barbone is detected in a neighborhood, they hasten to collect their animals and remove them to a more elevated region. It is known, moreover, that the organisms of the Pasteurella group remain virulent for a very long time in the mud of the marshes and in the slime of the streams.

Rôle of the bacteriophage in the disease

In Cochin-China barbone is always present in sporadic form causing each year numerous small epizootics which remain localized in individual villages. A localized epidemic observed in Long Huu in the Province of Gocong may serve as an example.

From May 5 to 13, 1920, seventeen buffaloes died:—on May 5, one; May 7, three; May 8, two; May 9, one; May 10, two; May 11, four; May 12, three; and May 13, one. Then the epizootic stopped and not a single case was detected during the next six months.

Specimens of the feces of four of these animals were collected, either before death or from the cadaver. None contained a bacteriophage active for the bacterium of barbone. On May 13 specimens of feces were collected from healthy animals, as follows:

First. From a buffalo in a stable where two animals had died, one on May 12, the other on May 13.

Second. From three buffaloes in a stable where one had died on May 5.

Third. From two buffaloes in a stable where two had died, one on May 8, the other on May 11.

Fourth. From four buffaloes in a stable which had not been invaded.

Fifth. From one buffalo, living alone in a stable located at a distance of about five kilometers from the village of Long Huu.

Sixth. From eight buffaloes in the surrounding villages, from eleven to nineteen kilometers distant.

Of all the specimens, those in the first, second, third and fourth groups gave a bacteriophage of weak or average activity (+ or ++) for the bacterium of barbone. An active bacteriophage was not found in the specimens from groups 5 and 6.

Again on May 19 specimens were collected in Long Huu, as follows:

First. From the buffalo which had furnished specimen no. 1 on May 13.

Second. From two buffaloes living in a stable where three had died from May 7 to May 12. These specimens all gave a bacteriophage moderately virulent (++) for the bacterium of barbone.

The animals which resisted, therefore, showed in their intestine a bacteriophage virulent for the pathogenic bacterium.

The epizootic does not always remain localized in a village. At times it spreads rapidly from village to village and within a few days will extend over a very considerable territory. It is rarely possible to determine the primary focus, so great is the speed with which it spreads. The mortality then becomes considerable, the losses often amount to tens of thousands of animals, as has been observed many times in China, in British India, and in the Dutch East Indies. Sometimes even, as actually happened in Java, the buffalo, as a race, is practically eliminated.

In the first two weeks of June 1920 the epizootic became general in the Province of Bac Lieu and in certain parts of the adjacent provinces (western Cochin-China). It was possible to examine the blood of eleven animals which died in widely scattered parts of the area invaded, and in all the bacterium of barbone was found in considerable quantity.[9] The epizootic died out during the first fortnight of July. It had persisted for a month, killing a third of the animals in the district.

The region of Thoi Binh was particularly affected, the loss amounting to more than fifty per cent of the buffaloes in the locality. From July 8 to 13, at the time when the epizootic was disappearing (the last animal to be affected died on July 12), twenty specimens of feces were collected. These were taken from buffaloes which had resisted the infection and which at no time showed any evidence of the typical symptoms of the disease. All of the animals examined lived on the farms of the village of Thoi Binh or in the neighboring hamlets within a radius of fifteen kilometers.

Tests for the virulence of the intestinal bacteriophage against the bacterium of barbone gave the following results:

[9] Bacteriological diagnosis is easy, even if the only available material is some blood or a fragment of an organ taken without any special precautions in the field, as is usual in such countries. Even if the specimen is some days old it is only necessary to smear it over the shaved skin of a rabbit. If the bacterium of barbone is present the animal will die within 24 hours, and the organism will be found in pure culture in the blood, from which it may be readily isolated. This is also the best method for detecting the bacterium in soil or in fecal material.

FARM	MORTALITY	THE LAST ANIMAL DIED ON	NUMBER OF ANIMALS WHICH RESISTED	VIRULENCE OF THE BACTERIOPHAGE
Ngau 1	3	July 7	10	++
Ngau 2				+
Ngau 3				+++
De	2	June 10	1	+
Doi 1	5	June 28	4	++++
Doi 2				+++
Lanh	9	July 2	4	++
Tran	1	July 4	2	++++
The	3	July 11	1	++++
H. v. Chanh	2	July 2	2	++++
Hien	0		4	++++
Du	0		6	+
Sam	2	July 2	8	++
P. v. Chanh	6	June 30	8	++++
Cu	1	July 2	5	+++
So	3	July 6	5	++
No	5	June 30	10	+++
Phuc	8	July 3	4	++++
Gia	8	June 29	3	+++
Man	1	June 30	3	++

From this it appears that the intestinal bacteriophage is endowed with virulence for the bacterium of barbone in all the buffaloes which the disease had spared.

In the course of different trips across Indo-China, I collected forty-one specimens of feces from buffaloes, each specimen collected in a different village in which no buffaloes had died of barbone for at least two years. In only three of these specimens could a bacteriophage active for the bacterium of barbone be demonstrated, and in these cases it was weak (+). Nevertheless, the intestinal bacteriophage was present in all; but although it was active for one or another of the intestinal organisms, its virulence was weak or lacking for the bacterium of barbone.

We will see later, on the contrary, that in a contaminated area at the time when the epizootic dies out, the intestinal bacteriophage of all of the buffaloes which escaped the disease is virulent for the bacterium, the causative agent of the epizootic. We find here, then, the same facts as in the previous disease studied;

that the protection of the body in the case of barbone, a septicemic disease, is assured by the bacteriophage.

In the buffaloes of a region ravaged by the disease the bacteriophage preserves for a very long time its virulence for the pathogenic bacterium. This, the following example shows.

In November, 1919, a localized epizootic of barbone occurred among the buffaloes of the village of Phuoc Thien (Province of Bien Hoa). On a farm having twenty-one buffaloes seven died —two adult animals and five aged from one to two years. The disease died out, or to speak more correctly, after this, two animals recovered one after another. On the 12th of the following April, that is to say, five months later, specimens of the feces of eight of the surviving animals were collected. All contained a bacteriophage active for the bacterium of barbone (six were ++, two were +).

Two specimens of the mud of a water-hole where the animals were accustomed to remain immersed up to the neck during the hottest hours of the day were also examined. In both a bacteriophage virulent (++) for the bacterium of barbone was found. The destruction of the pathogenic bacterium in the external medium must often be effected by the bacteriophage, for it is certain that if the bacterium of barbone has once been introduced into a water-hole by a sick animal the bacteriophage present there must destroy it. Furthermore, this fact shows one of the modes of "contagion" of the active bacteriophage. A single buffalo, in the intestine of which the bacteriophage has acquired a virulence for the pathogenic bacterium, is sufficient to "contaminate" all the herd which frequent the water-hole. Localized epizootics are of short duration, but in spite of this we find that the pathogenic bacterium persists for several months in the external world and that their ingestion by buffaloes is frequent, since the virulence of the bacteriophage maintains itself against this bacterium. The repeated ingestion of a bacterium is, as we have seen, essential for the permanence of the virulence of the bacteriophage toward this bacterium. The epizootic dies out, not because of an absence of pathogenic bacteria but because of the presence of a virulent bacteriophage in the intestine of all exposed animals.

All of the observations are therefore comparable, whether they deal with avian typhosis or with barbone in the buffalo. These epizootics of very different nature were investigated intentionally, that the general nature of the rôle of the bacteriophage in immunity might be the better established.

One may at first be quite astonished that the intestinal bacteriophage, whose rôle can easily be conceived in infections with intestinal manifestations, constitutes a defense of the organism in septicemias. In reality, whatever may be the infection, the pathogenic bacterium always gets into the intestine. Let us take a localized disease, cerebrospinal meningitis, for example. We know that the initial symptom is a rhino-pharyngitis and that even healthy subjects who have been in contact with a patient often carry the specific germ in the nasopharynx. There can be no doubt but that a fair number of the meningococci present in the rhino-pharynx are swallowed and pass into the intestine. It is needless to insist on this, that, aside from a few rare exceptions to which we will later return, whatever may be the disease under consideration, the portal of entrance of the virus is either the buccal route or by way of the respiratory tract. In either case the ingestion of organisms is, it might be said, obligatory. The pathogenic bacterium is always at some time in contact with the intestinal bacteriophage, this organism therefore is thus able to adapt itself to the bacteriophagy of the bacterium and to acquire a virulence.

In the particular case of barbone the pathogenic bacterium is found freely disseminated through the exterior world in contaminated regions. In an epizootic zone I have been able, in two different trials, to isolate it from the mud of a marsh where the buffaloes were accustomed to bury themselves. This is but natural since the bacterium of barbone is found in the intestinal tract of sick animals or of those which have succumbed. The ingestion of the pathogenic bacterium by the animals which remain immersed for whole hours in a mire containing these organisms is necessarily frequent. If the animal which ingests them has an erosion at any point in the digestive tract it is susceptible to infection. Otherwise the bacteria reach the intestine and come within the range of the intestinal bacteriophage which

can then acquire a virulence for the virus. If this takes place the animal is thenceforth protected from the infection and becomes a carrier of the virulent bacteriophage. A diseased animal propagates his disease; an animal in a resistant condition propagates his immunity.

BUBONIC PLAGUE

Through a lack of favorable circumstances it has not been possible to follow the evolution of the intestinal bacteriophage in man affected with plague. The few cases that have been examined have all been fatal, and at no time could the intestinal bacteriophage be shown to have the least virulence for *B. pestis*. The activity in these cases remained restricted to *B. coli*. However, the stools of two convalescent individuals have been secured and examined. According to the physicians treating the cases the material was collected on the sixth and the eleventh days after the beginning of convalescence. Examination showed, in the first case, a bacteriophage of average virulence (++), and in the second case, one of feeble virulence (+) for *B. pestis*. The virulence of the first of these strains has been enhanced *in vitro* and the bacteriophage has been maintained in culture.

An attempt was made to find a bacteriophage active against this bacillus in the feces of twenty-two natives living in regions free of plague, but in no case could a strain be isolated. However, in view of the particular mode of infection in bubonic plague the study of its propagation in man offers only a matter of secondary interest, at least from the epidemiological point of view. We know that an epidemic of plague in man is only consequent to an epizootic among rats. That which it is interesting to study is, therefore, the epizootic, the primary cause of the epidemic. In order to attain a correct interpretation of results it is essential to follow the natural order of things. From the point of view of man the epidemic is obviously the important fact; from the point of view of nature this is but a secondary incident, for if we were able to suppress the epizootic the epidemic would cease spontaneously.

From what we actually know about the epidemiology of plague, it results that all of the rats living in a city where there has been

a case of plague in man are the animals which have resisted the contagion, either because they were infected and recovered, or because they remained unaffected. I have then, investigated the virulence of the intestinal bacteriophage of the rat toward *B. pestis*.

First. Twenty-one specimens of the excrement of rats taken from towns in Indo-China free of plague were examined. The intestinal bacteriophage was found, active against one or another of the intestinal bacteria, but it never showed any virulence whatever for *B. pestis*.

Second. A small epidemic of plague (eleven fatal cases) occurred in the village of Bac Lieu, in the eastern part of Indo-China, during July, 1920. On the following 6th of November I procured in this town four specimens of the excreta of rats, each specimen composed of some dozens of particles, and certainly derived from several individuals. The tests for virulence against *B. pestis* gave the following results:

Specimen derived from a granary	++
Specimen derived from the embarkment quay	+++
Specimen derived from a decorticating mill	+++
Specimen procured in the house of a native	++++

Those rats which have survived an epizootic, therefore, harbor in their intestine a bacteriophage possessed of a high virulence for *B. pestis*.

Plague has existed in the form of sporadic cases in the region of Phantiet, in southern Annam, for about twenty years. I obtained specimens of the excrement of rats in the infected villages, each specimen being composed of the feces derived from several animals. The results of the tests for the virulence of the intestinal bacteriophage in these specimens were:

Village	*Virulence*
Thien Duc	++
Hung Long	+
Duc Hang	+++
Duc Thang	++
Tri Long	++
Phu Tay	+++
Cu Long	+

The results are thus identical with those secured at Bac Lieu, although the virulence seems to be somewhat lower, but this can only be of relative importance since in the present case each specimen was composed of the excreta taken from several rats; the results then, indicate only an average.

Is the bacteriophage present in all of the rats of an infected region or only in a certain number? At Phantiet I collected the excrement of six young rats, according to their weight, aged from three to four weeks. Examination showed that four of the specimens contained a bacteriophage active for *B. pestis* (+) while two did not. These last two animals were therefore susceptible to plague.

From the results given above one may conclude that, as for avian typhosis and for barbone, the cause of the resistance against *B. pestis* is the presence in the organism of a bacteriophage possessing a virulence for this bacillus.

How is the adaptation effected in the case of *B. pestis?* At different times it has been noted that the bacillus has been found in the intestinal contents of victims of plague. Thus, it is possible for them to be disseminated by the feces throughout the external world where they may again be ingested. The bodies of dead rats constitute another mode of dissemination. These bodies are often devoured by the surviving rats and this extends the infection. In those animals which resist and which are infected the intestinal bacteriophage is maintained virulent for the pathogenic bacillus. But observation and direct experimentation have shown us that a bacteriophage is only possessed of a virulence for a bacterium when the ingestions of this bacterium are frequent. The permanence of the virulence of the intestinal bacteriophage of the rat against the plague bacillus indicates the persistence of this bacillus in the external world, at least for several months after the last human case has taken place. Moreover, the revival of the epidemic each year in certain localities, Bac Lieu for example, shows that it can not be otherwise.[10]

[10] Demonstration of the presence of a bacteriophage active for *B. pestis* in the rats of a locality would in certain cases be very useful for it would indicate the presence of the bacillus in the exterior world and the possi-

FLACHERIE

A few experiments have been made on this disease, but only for the purpose of determining if defense against infection in invertebrates is also assured by the bacteriophage.

In a breeding-place in Cochin-China a certain number of silk worms died of a disease presenting all of the characteristics of flacherie. Examination of the excreta of the sick worms, as well as of the cadavers, showed the presence of a cocco-bacillus, Gram-negative, which was not present in the dejections of healthy worms. The ingestion, on mulberry leaves, of some of the culture of this cocco-bacillus reproduced the disease; eleven out of twelve worms dying in from six to eleven days after the infecting feeding.

Three filtrates were prepared from the excreta of healthy worms living in the baskets where the affected worms were found. These three filtrates contained a bacteriophage of moderate or high virulence (++, +++, +++) for the cocco-bacillus. On the other hand, two filtrates were prepared, the one with the intestinal contents of a sick worm, the other with the intestinal contents of a worm which had died of the infection. Neither contained a bacteriophage active for the coccobacillus.

These experiments have not been carried further, since the desired end had been attained. They were adequate to show that the facts observed in infectious disease in mammals were reproduced in an infectious disease of an invertebrate. From this it seems logical to conclude that the defense of the organism by the bacteriophage must constitute a general fact throughout all animals.

bility of a renewal of the epidemic. Such a demonstration might also be useful in establishing a retrospective or doubtful diagnosis. Suppose a few suspicious deaths have occurred in a group some time previously. The presence in the rats of the neighborhood of a bacteriophage showing a virulence for *B. pestis* would eliminate all doubt; the deaths were due to plague. Or, the question of the nature of a epizootic among the rats may be in question. Was the mortality due to plague? The demonstration of a bacteriophage active for *B. pestis* either in the dead rats or in those that have survived provides the answer.

CONCLUSIONS

We may limit ourselves for the moment to the following points:

Whatever may be the disease considered, the picture remains the same; when a pathogenic bacterium is introduced into an organism, one of two situations develops:

First. The intestinal bacteriophage shows an activity for this bacterium, the latter is destroyed before it can develop, and disease does not appear.

Second. The intestinal bacteriophage is inactive, the bacterium develops, and disease results.

In the course of the disease one of two things may happen:

First, the bacteriophage in contact with the pathogenic bacterium may acquire a virulence, or

Second. The bacterium may acquire a virulence, in other words, may become resistant to the action of the bacteriophage.

The vicissitudes in the struggle between these two factors are reflected in the condition of the infected individual. Convalescence begins at the moment when the virulence of the bacteriophage is sufficient to give it, definitely, the upper hand. The disease has a fatal outcome if the bacteriophage is inactive as a result of unfavorable conditions, or if the bacterium is able to acquire a refractory state. This last situation appears to be very infrequent, at least, in the diseases studied.

In epidemics we find a large scale reproduction in a community of individuals of the struggle which takes place in a single individual between the ultramicrobe and the bacterium.

The bacteriophagous ultramicrobe is transmissible from one individual to another just as is the bacterium itself. The history of an epidemic is, in the last analysis, the story of an infection with two microorganisms. The epidemic ceases at the moment when all susceptible individuals harbor a bacteriophage active for the causative organism of the epidemic. Either the bacteriophage has acquired virulence in the body of the individual who harbors it, or this individual has been "contaminated" by a bacteriophage which has acquired a virulence in another individual for the specific bacterium involved.

CHAPTER II

The Bacteriophage in the Healthy Individual

The Bacteriophage in Man. The Bacteriophage in the Horse. The Bacteriophage in Fowls. The Bacteriophage in Diverse Animals. Conclusions.

The experiments conducted on patients and on healthy individuals exposed to infection have shown that a resistance to the infectious agent accompanies the presence in the intestine of an ultramicrobial bacteriophage possessing a virulence for the causative bacterium. On the other hand, as I have shown in Part I of this monograph, there is but a single species of bacteriophage, capable, by adaptation, of acquiring virulence for the diverse bacteria which it attacks.[1]

These facts being true, a question quite naturally arises. Does this bacteriophage which acquires virulence for diverse pathogenic bacteria make its appearance only at the exact moment when it is needed? Or is it, indeed, a normal inhabitant of the intestinal canal? Examination of the feces of numerous individuals belonging to very varied species permits an answer to this question.

THE BACTERIOPHAGE IN HEALTHY MEN

Variations in the virulence of the intestinal bacteriophage in healthy men have been followed. To this end, in a first series of experiments, specimens of the stool were collected every fifteen days. The study has been limited to testing the activity of the ultramicrobe against the following bacterial species: *B. coli*, *B. dysenteriae* Shiga, *B. dysenteriae* Flexner, *B. dysenteriae* Hiss, *B. typhosus*, *B. paratyphosus A*, and *B. paratyphosus B*. Later

[1] The possibility of a germ being virulent for a great number of different beings is not an exception peculiar to the bacteriophage. It is only necessary to mention *B. tuberculosis*, to which but few animals are insusceptible.

where indicated, the tests were extended to other bacterial species of particular interest.

With the first examinations a weak activity (+), especially for *B. coli*, was not detected or remained doubtful, but when the tests were repeated after several months, using a more satisfactory technic, the bacteriophage was clearly demonstrated. As will be seen upon examining the table where the results are recorded (table 1), some of the examinations remained negative; the bacteriophage appeared to be absent. Would it have been the same if it had been possible to test the filtrate against all of the bacteria which may be found in the intestine? An answer to this question was sought. A specimen taken on July 1st was inactive toward the eight species of bacteria routinely employed, and it was tested against a different series of bacteria, selected at random. The filtrate showed a high activity for an organism of the Salmonella (hog cholera) group. When the same experimental tests were repeated on December 1st this filtrate was active for *B. enteritidis*.

These two examples are sufficient to show that the absence of the bacteriophage is only apparent. It must be remembered that the ultramicrobe is recognized only by its activity, and consequently its presence in a filtrate can be detected only by testing it against a bacterium for which it has a definite activity. On the other hand, it is not possible to carry out the examination on strains of all bacteria which may be found normally or occasionally in the intestine. For this there are several reasons, the chief being the difficulty of numbers, for there is no species of bacteria which may not be found in the intestinal tract at one time or another. We have seen further that certain bacterial species are not "homogeneous" as regards the bacteriophage. *B. coli* is of this group. Certain strains are attacked while others remain unharmed. It would then be necessary to make tests with all varieties of a single species; a new impossibility. Finally, it is known that there exist in the intestine certain bacteria, revealed by the microscope, which it is impossible to isolate and cultivate. May the bacteriophage not live in commensalism with these bacteria, with which they may form in the intestine "mixed cultures?" It may even be that the impossibility

of isolating these bacteria may be due solely to this phenomenon of commensalism, for we have seen that the agar on which mixed cultures are planted sometimes remains free of bacterial colonies.

TABLE 1

DATE	VIRULENCE OF THE INTESTINAL BACTERIOPHAGE FOR							
	B. coli	*B. dysenteriae*			Bacillus			Other organisms
		Shiga	Flexner	Hiss	Typhosus	Para A	Para B	
January 15.....	0	0	0	0	0	0	0	
February 1.....	+	0	0	0	0	0	0	
February 15	0	0	0	0	0	0	0	
March 1.....	0	0	0	0	0	0	+++	
March 15.....	+	0	0	0	0	0	0	
April 1.....	+	0	0	0	0	0	0	
April 15.....	+++	0	0	0	0	0	0	
May 1.....	+	0	0	0	0	0	++	
May 15.....	+	0	0	0	0	0	0	
June 1.....	0	0	0	0	0	0	0	
June 15.....	+	0	++	+++	0	0	0	
July 1.....	0	0	0	0	0	0	0	Salmonella+++
July 15.....	+++	0	0	0	0	0	0	
August 1.....	+	+	0	0	0	0	0	
August 15.....	+++	+++	0	0	0	0	0	
September 1	+++	0	0	0	0	0	0	
September 15 ...	+	+++	0	0	0	0	0	
October 1.....	+	0	0	0	0	0	+++	
October 15.....	+	+++	++	0	0	0	0	
November 1	+	0	0	0	0	0	0	
November 15 ...	+	0	0	0	0	++	0	
December 1.....	0	0	0	0	0	0	0	*B. enteritidis*++
December 15....	+	0	0	0	0	0	0	

However this may be, the table here given shows the results of the tests made on the normal man in question. The examinations are adequate to show that the bacteriophage is a normal inhabitant of the intestine (table 1).

During the course of this same year this individual showed at two different times, July 3, and September 26, slight intestinal

disturbances lasting some hours. The first time there was no obvious cause; the second attack followed a suspected meal taken in a village tavern. On each occasion specimens of the stools were examined on the following days. The results are recorded in table 2.

There can be no doubt that in the first case the cause was *B. dysenteriae* Flexner, and in the second *B. paratyphosus B.* Disease was aborted, the bacteriophage having quickly acquired virulence for the invading germ.

TABLE 2

DATE	B. COLI	B. DYSENTERIAE			BACILLUS		
		Shiga	Flexner	Hiss	Typhosus	Para A	Para B
July 4	+++	0	+++	++	0	0	0
July 5	++	+	+++	+	0	0	0
July 6	+++	+	++	+	0	0	0
July 7	++	0	++	0	0	0	0
July 8	+	0	0	0	0	0	0
September 27	+++	++	+	+	+	0	++++
September 28	+++	+	0	0	0	0	++++
September 29	+++	0	0	0	0	0	++
September 30	++	0	0	0	0	0	+++
October 1	+	0	0	0	0	0	+++
October 2	+	0	0	0	0	0	+
October 3	+	0	0	0	0	0	0

We will complete this section by giving the results of tests made on the stools of three normal persons (aged 39, 22, and 17 years) during the period of the 15th to the 30th of July. Only the bacteria against which the bacteriophage showed a virulence in each of the three persons designated by the numbers I, II, and III, are recorded (table 3).

It is unnecessary to multiply examples. All of the experiments which have been conducted have given comparable results. One conclusion stands out; the bacteriophage is a normal inhabitant of the human intestinal canal.

THE BACTERIOPHAGE IN THE HORSE

Sixty-two specimens of manure derived from horses living both in cities and in the country, in France and in Indo-China, have been examined and all contained an active bacteriophage. A list of the animals is given, and the results of the examination are recorded in table 4.

No. 1. Horse No. 21 of the Pasteur Institute. This horse was used in the production of anti-dysentery serum. The examination was made three days after the injection of Shiga toxin.

TABLE 3

DATE	I	II	III
July 15	C+	C+++	0
July 16	C+	C+++	C+++
July 17	0	C+	0
July 18	C+++ H+++	0	0
July 19	C+++ H++	0	C++ B+
July 20	C++ H+++	0	C+ B+
July 21	C++ H+	C+	0
July 22	0	0	0
July 23	0	0	C+
July 24	C+++	0	0
July 25	C++	0	0
July 26	0	0	0
July 27	C++ Sh.+++	C++	C+++
July 28	C+ Sh.+	0	0
July 29	0	C+	0
July 30	C+	C+	0

C = *B. coli;* H = *B. dysenteriae* Hiss; Sh. = *B. dysenteriae* Shiga; B = *B. paratyphosus B.*

No. 2. Horse No. 21 (above), tested ten days later.

No. 3. Horse No. 21 (above), tested four months later, the examination being made 48 hours after a toxin injection.

No. 4. Horse No. 114. Used in the production of Shiga anti-dysentery serum.

No. 5. Horse No. 18. Used in the production of Shiga anti-dysentery serum.

No. 6. Horse No. 18. (above) tested four months later. The specimen was collected 48 hours after the injection of Shiga toxin.

No. 7. Horse No. 64. This horse had received injections of atoxic dysentery bacilli—Flexner and Hiss—for two years.

No. 8. Horse No. 65. This horse had received injections of atoxic dysentery bacilli—Flexner and Hiss—for two years.

TABLE 4

HORSE	B. COLI	B. DYSENTERIAE			BACILLUS			B. GALLINARUM
		Shiga	Flexner	Hiss	Typhosus	Para A	Para B	
1	++	++++	++	++	0	0	0	−
2	+	+	+	0	0	0	0	−
3	+	++	0	0	++	+	+	−
4	++	++	+	+	0	+	++	0
5	++	+++	+++	++	0	0	0	0
6	+	0	+++	+++	0	0	0	0
7	++	++++	++++	++	0	0	0	−
8	++	+	++++	++++	0	0	0	−
9	++	++++	++	++	0	0	0	−
10	++	0	++	++	0	0	+	−
11	+	0	+	++	0	0	0	0
12	++	+++	+	++	0	0	+	0
13	++	++	++	+	+	0	0	0
14	++	++++	++	++	0	0	0	0
15	0	++++	++	+++	0	0	0	0
16	++	++++	++++	++++	++	+++	++	++
17	++	+++	++	++	+	++	0	++
18	+++	++	+++	+++	+++	+++	++	+++
19	++	++++	+	+	0	0	++	0
20	0	++	0	0	0	0	0	0
21	+	+	+	0	0	+	++	0
22	++	0	0	0	0	0	++	0
23	++	+++	++	++	0	0	++	0
24	+	++++	+++	++	++	0	+	0
25	+	++	0	0	0	0	0	0
26	+++	+++	++	++	0	0	+++	0

No. 9. Horse No. 68. This horse had received injections of atoxic dysentery bacilli—Flexner and Hiss—for two years.

No. 10. This horse was receiving injections of *B. anthracis.*

No. 11. This horse was receiving injections of *B. anthracis.*

No. 12. A carriage horse in Paris.

No. 13. A carriage horse in Paris.

No. 14. A carriage horse in Paris.

No. 15. The same animal as No. 14 (above) but tested four days later.

No. 16. A farm horse on a farm where avian typhosis was present.

No. 17. A farm horse on a farm where avian typhosis was present.

No. 18. A farm horse on a farm where avian typhosis was present.

No. 19. A race horse at Chantilly.

No. 20. A race horse at Chantilly.

No. 21. The same animal as No. 19 (above) but tested eight days later.

No. 22. The same animal as No. 20 (above) but tested eight days later.

No. 23. A carriage horse at Saïgon.

No. 24. A carriage horse at Saïgon.

No. 25. A saddle-horse at Nha-Trang (Annam).

No. 26. A saddle-horse at Phantiet (Annam).

There is no point in adding to this list; the thirty-six other specimens gave entirely comparable results.

Incidentally, horses No. 19 and No. 20, were examined to see if the bacteriophage presented a virulence for various other bacteria, including the following organisms:

1. A cocco-bacillus (?) isolated from the nasal mucus of a horse in the same stable which showed the evening before an elevation of temperature:

Horse No. 19 (++), horse No. 20 (++).

2. A cocco-bacillus isolated by Césari from the blood of a horse slaughtered in the abattoir of Vaugirard:

Horse No. 19 (+) horse No. 20 (++).

3. Salmonella (hog cholera):

Horse No. 19 (++), horse No. 20 (++).

4. *B. enteritidis:* Horse No. 19 (0), horse No. 20 (+).

These results show that at a single time the bacteriophage may show a virulence for a large number of bacteria. It is significant that only in horses Nos. 16, 17, and 18, which lived in an environment contaminated by *B. gallinarum*, did the intestinal bacteriophage show a definite virulence for this bacterium.

Examination was made of twenty-three specimens of serum, of clot remaining after the decantation of the serum, and of the leucocytic layer on top of this clot, taken from horses harboring in their intestines a bacteriophage active for *B. dysenteriae*. In no case was a bacteriophage found. In all instances the specimens of blood were collected about two weeks after the last injection of toxin or of bacilli. All the specimens of blood examined came from horses furnishing anti-dysentery serum. It was therefore not determined whether the bacteriophage may not pass into the circulation immediately after the injection, especially when living bacteria are used. As a matter of fact the passage of the intestinal bacteriophage into the circulation has been observed in the rat, and in the fowl in cases of septicemia. In all cases the demonstration of the presence, it might be said constant presence, in the excreta of the horse of a bacteriophage active for the Shiga bacillus, and the absence of this bacteriophage in the blood, shows in an unquestionable manner that the intestine is the only locality where the bacteriophage normally grows. This fact alone is sufficient to demonstrate the error of the conception of Bordet, who has advanced the hypothesis that the bacteriophage is of leucocytic origin.

THE BACTERIOPHAGE IN THE FOWL

I have made seventy examinations of the excreta of fowls, and have tested the bacteriophage for virulence against the eight bacterial strains selected. It is needless to give all the results since they were all of the same nature. As examples, the results of only one or two tests in each lot will be given (table 5).

Nos. 1 and 2 represent chickens living in France in regions free of avian typhosis (12 specimens examined).

Nos. 3 and 4 represent healthy fowls living in regions where avian typhosis was present (19 other examinations carried out on the eight test bacteria gave comparable results, particularly as regards *B. gallinarum*).

Nos. 5 and 6 represent chickens which had recovered from avian typhosis (4 tests made).

Nos. 7 and 8 represent chickens which died of avian typhosis (8 other tests gave similar results). The bacteriophage was present but was not virulent for the pathogenic bacillus.

Nos. 9 and 10 were chickens living in Cochin-China in regions free of both avian typhosis and barbone.

Nos. 11 and 12 represent chickens living in Cochin-China in areas where barbone was present but free of avian typhosis (11 tests, all essentially the same).

Nos. 13 and 14 were chickens in France living in regions where avian typhosis was present.[2]

TABLE 5

NUMBER	B. COLI	B. DYSENTERIAE			BACILLUS			B. GALLINARUM	BACT. BARBONE
		Shiga	Flexner	Hiss	Typhosus	Para A	Para B		
1	0	0	+	+++	0	0	0	0	−
2	++	++	0	0	+	0	+	0	−
3	+	+++	+++	+++	+	+	++	++	−
4	++	+	+	++	++	0	+	+	−
5	+++	++++	+++	+++	++	++	++	++++	−
6	++	+++	+++	+++	0	+	+++	+++	−
7	+	++	0	0	0	0	++	0	−
8	0	0	++	+	0	0	+	0	−
9	++	+++	+	++	0	0	0	0	0
10	+	+	0	+++	0	0	++	0	0
11	++	+++	++	++	0	0	++	0	+
12	0	+	0	+	++	+	++	0	++
13	+++	++++	+++	+++	+	++	++	++	−
14	++	++	++	++++	0	0	+++	++	−

THE BACTERIOPHAGE IN DIVERSE ANIMALS

Examinations have been made of the excreta of the following animals (table 6).

No. 1. A monkey, confined in a cage in Paris.

Nos. 2 and 3. Cats in Paris.

Nos. 4 and 5. Cattle living on a farm where avian typhosis was present.

[2] I would recommend that bacteriologists desiring to procure strains of the bacteriophage investigate principally the excreta of horses and chickens, particularly at the beginning of their work. It is from these animals that it is most easy to isolate strains of the bacteriophage having a high activity when taken from the body. These excreta are, moreover, more readily procured than the feces of convalescents.

Nos. 6 and 7. Cattle in France, in a region free of epizootic diseases.

Nos. 8 and 9. Steers in Cochin-China, living in regions free of epizootics (42 other comparable tests).

TABLE 6

NUMBER	B. COLI	B. DYSENTERIAE			BACILLUS			BACTERIUM OF BARBONE	B. GALLINARUM
		Shiga	Flexner	Hiss	Typhosus	Para A.	Para B		
1	+	++	0	0	0	0	0	−	−
2	0	+	++	+	0	0	0	−	−
3	+	0	+	+	0	0	0	−	−
4	0	0	0	+++	0	0	0	−	+
5	++	++	+	+	0	0	0	−	++
6	+	++	++	0	0	0	0	−	0
7	0	++	0	0	0	0	0	−	0
8	++	+++	++	+	0	0	++	−	0
9	+	0	+	0	0	0	0	−	0
10	+	++++	++	0	0	0	0	0	−
11	++	0	+++	0	0	0	0	0	−
12	+++	+	0	0	0	0	0	++	−
13	+++	++	++	0	0	0	0	+++	−
14	++	+++	++	0	0	0	+	0	−
15	+++	+	+	0	0	0	0	0	−
16	+	+++	0	+	0	0	+	+	0
17	++	+	0	0	0	0	0	+	0
18	+	0	0	0	+	0	++	−	0
19	+	++	0	+	0	0	0	−	0
20	++	++	+++	+++	0	0	+++	−	++
21	+++	+	0	+	+	+	++	−	+
22	+	++	+	+++	0	0	0	−	−
23	0	+	0	0	0	0	0	−	−
24	++	0	+	0	0	0	0	−	−
25	++	0	0	0	0	0	0	−	−

Nos. 10 and 11. Buffaloes living in regions free of barbone (14 other comparable tests).

Nos. 12 and 13. Healthy buffaloes living in regions where barbone was present (24 other comparable tests).

Nos. 14 and 15. (for comparison) Buffaloes sick (14) or dead (15) of barbone. Eight other tests have been made; five were

comparable to those cited. In three others a bacteriophage was not found; if it was present it was inactive for the eight test organisms.

Nos. 16 and 17. Swine in Cochin-China, in a barbone area.

Nos. 18 and 19. Swine in Paris.

Nos. 20 and 21. Swine in France, on a farm infected with avian typhosis (4 other comparable results).

Nos. 22 and 23. Rabbits living in cages at the Pasteur Institute (4 other comparable findings).

Nos. 24 and 25. Goats in Paris.

CONCLUSIONS

In brief, in all the healthy animals examined the presence of a bacteriophage possessing virulence for one or another of the intestinal bacteria selected as test organisms has been demonstrated.

These examinations show that in an epizootic area the intestinal bacteriophage of refractory animals is generally possessed of virulence for the bacterium causing the epidemic; on the contrary, this is never observed outside of the foci of infection.

The activity of the bacteriophagous ultramicrobe towards a given bacterium can only be explained as the result of growth at the expense of this bacterium. Tests of the virulence of the ultramicrobe in animals inhabiting an epizootic region or living in an area free of the infection, against the bacterium considered as the cause of the disease, are, among other proofs, conclusive in this respect. The experiments *in vitro* are in accord with the facts observed in animals. All the facts agree in showing that in the body the bacteriophage ceases to be active for a bacterium a few days after the destruction of this bacterium. With animals, the ingestion of typhoid, paratyphoid, and especially, dysentery bacilli, must be extremely frequent, since in animals the intestinal bacteriophage is, except in rare instances, virulent for one or another of these organisms.

The sum total of the results suggests that the bacteriophage possesses "co-virulences" or "accessory virulences" extending to organisms belonging to the same group as the invading bacillus. For example, a bacteriophage increasing its virulence for the Shiga strain of *B. dysenteriae* must at the same time, although to a less

degree, attack the bacillus of Flexner, or that of Hiss, or both at once. It is difficult to explain in any other manner the simultaneous appearance of virulence for several bacilli of the same group. These co-virulences extend generally to the bacillary species which show among themselves the phenomenon of co-agglutination.

With man, also, much less exposed to contagion because of his mode of life, the activity of the bacteriophagous ultramicrobe for the typhoid, paratyphoids, and dysentery bacilli is very frequent. Each time that it occurs it can only be the indication of an incipient infection, which usually passes undetected. The bacteriophage, as a result of its rapid adaptation destroys the invading bacilli before they can multiply.[3]

The ultramicrobial bacteriophage is a normal inhabitant of the intestine of all animals. Furthermore, as we have seen, it has a great vitality. Thanks to its minuteness it is able certainly to filter through soils which arrest bacteria. Everything shows that it must be extremely widely disseminated in the external world. Everything which at any time may be contaminated by the excreta of any animal must contain it. It must be particularly abundant where there are living organisms; in the soil, in rivers, and in the ocean.[4] And everywhere it must be the principal factor in the destruction of bacteria.

[3] It seems then, even in animals considered refractory, that there occurs at times a delay in the adaptation of the bacteriophage. This is noted, for example, in cases of dysentery (Shiga bacillus) in horses in tropical countries.

[4] I have isolated a strain of the bacteriophage active for *B. coli* from a specimen of sea-water taken off from the estuary of Mekong, and a second taken from the Mediterranean off Marseilles. I was unable to isolate a strain from a specimen of water from the Indian Ocean at a point approximately 60° East longitude and 10° North latitude. Dumas has isolated strains from a specimen of garden soil and from the tap-water in Paris. Beckerich and Hauduroy, at the Institute of Hygiene at Strasbourg, have isolated a number of strains active for *B. coli*, for *B. typhosus*, and for dysentery organisms, from different specimens of soil, from the water of the Ill, and from Rhine water after sand filtration.

These findings allow us to draw an important conclusion, which has a bearing on hygiene. The bacteriophage exercises a preponderant rôle in the defense of the organism. It is therefore of great interest that drinking

CHAPTER III

Immunization by Means of the Bacteriophage

Immunization against Avian Typhosis. Immunization against Barbone. Immunization against Dysentery. Conclusions.

The single test scientifically acceptable for a theory of immunity can be furnished only by the reproduction of this immunity in an animal naturally susceptible. That is, in placing this animal by experiment in the condition to which immunity is attributed.

A strain of the bacteriophage active for a given bacterium can be isolated and cultivated *in vitro* indefinitely at the expense of this bacterium, thus maintaining its virulence. In this way any desired amount of culture can be obtained.

If the theory of immunity by the bacteriophage which we have deduced from the investigations made on natural disease is correct we should be able at will to reproduce all the phenomena leading to recovery, provided there do not exist at the time of intervention organic lesions incompatible with life. We should likewise be able to place the exposed individual in the same refractory state enjoyed by the person who has passed through the epidemic period unaffected. It is for this reason that up to the present time I have confined myself almost entirely to a study of the rôle of the bacteriophage in animals, since these alone permit of experimental confirmation.

water should contain the greatest possible number of bacteriophagous ultramicrobes virulent for the agents of contagious diseases. It is certain that to obtain such waters, containing at once the greatest possible number of virulent ultramicrobes and the smallest possible number of bacteria it is necessary to use filtered river water and not stored waters. As potable waters the first are at the same time superior, from both the hygienic and economic points of view.

Attention may be called to the fact that in certain cases a search for a bacteriophage virulent for a given bacterium may be of some significance in hydrological investigations.

The immunization experiments by means of cultures of the bacteriophage have been conducted in two different ways:

a. In an epizootic area of avian typhosis, where there would be an immunization against natural infection; and

b. In a non-infected area, as in barbone, where the results could be checked against experimental controls. These last experiments have shown clearly the exact conditions under which immunization by means of the bacteriophage can be carried out.

IMMUNIZATION AGAINST AVIAN TYPHOSIS

Further mention need not be made of the immunization experiments conducted in the laboratory, which have been discussed in the chapter dealing with the phenomena observed in the course of epizootics of avian typhosis.

Immunization experiments in a region where the epizootic was present, as in the case of avian typhosis, presented an especial difficulty, or rather, a complication. It has been mentioned that aside from the typical typhosis, due to *B. gallinarum*, there are several varieties of paratyphoses, each caused by a particular species of bacterium. The differences which these bacterial species present from the biochemical and agglutinative points of view and which serve to differentiate them are of no particular significance from the point of view of this study. In so far as the action of the bacteriophage is concerned for each of these, a strain of bacteriophage having an extreme virulence (++++) for *B. gallinarum* possesses the same activity for all the French and American strains as well as for *B. jeffersonii*. The activity is less pronounced for *B. pullorum A*, still less for *B. pullorum B*, and is lacking for *B. pfaffi* and for *B. rettgerei*. With such a strain of bacteriophage the reactions are: *B. gallinarum* ++++, *B. jeffersonii* ++++, *B. pullorum A* ++, *B. pullorum B* +, *B. pfaffi* 0, and *B. rettgerei* 0. Inversely, a strain of bacteriophage secured from fowls resistant to paratyphosis due to *B. pfaffi* (focus at Trainel, Aube) had the following virulences: *B. gallinarum* 0, *B. jeffersonii* 0, *B. pullorum A* 0, *B. pullorum B* +, *B. pfaffi* ++++, and *B. rettgerei* 0.

The immunization experiments thus become singularly complicated, particularly since both typhosis and the paratyphoses

may be found in the same areas, as is also true for human enteric infections. In routine practise the solution is simple; it is sufficient to immunize the poultry with a mixture of cultures of different strains of the bacteriophage active against the diverse pathogens, the causes of typhosis and the paratyphoses. In the preliminary investigations this was not possible, for the differences between the different diseases had not been recognized when I first undertook this study. The different bacilli, the agents of the paratyphoses, had been studied in the United States but their simultaneous presence in foci of typhosis had not been noted. And in so far as *B. pfaffi* is concerned, discovered by Pfaff in an epizootic in Vienna, it had not then been incriminated as capable of producing disease in the Gallinaceae. These facts have only been disclosed gradually in the course of these investigations.

The cultures of bacteriophage used in the immunization experiments were prepared in the following manner:

A culture of *B. gallinarum*, in Martin bouillon, aged nine or ten hours, that is, very young but showing a definite turbidity, is inoculated with a bacteriophage isolated from the excreta of a recovered chicken and possessing a high virulence for the pathogenic bacillus. After about 12 hours the bacterial lysis is completely finished and the bouillon is perfectly limpid. This culture is filtered through a bougie[1] and distributed into ampoules which are sealed.

The dose employed for immunization has been in all cases 0.5 cc., given subcutaneously. The point of injection is of no importance for the slightest local or general reaction has never been observed.

Experiment I. The following experiments were conducted in 1919 and 1920 in the neighborhood of Agen with the assistance of M. Lambert, D.V.M.

Barnyard 1. The epizootic began in August, 1919. By October 2, 110 of 160 fowls had died. The 50 survivors, of which 5 were already affected, were inoculated with the culture of bacteriophage. The 5 sick chickens

[1] We have seen that whatever may be the virulence of the inoculated bacteriophage one may always obtain secondary cultures in a certain number of tubes. Filtration is thus essential.

recovered and the epizootic stopped abruptly and definitely on the same day as the immunization.

Barnyard 2. The epizootic began on about August 20. By October 6, 120 of 200 fowls had died. The 80 survivors, of which 7 were sick, received an injection of the antigallinarum bacteriophage. The 7 recovered; the epizootic immediately and permanently disappeared.

Barnyard 3. The epizootic began October 10. By the 15th, 21 fowls had died. The 130 that were alive, of which 8 were already sick, were inoculated. The 8 recovered and the epizootic disappeared from the day of the inoculation.

Barnyard 4. The epizootic began on about November 15. By December 1, 26 of 51 fowls were dead. The 25 survivors, among which were 4 which were infected, were inoculated. One of the sick animals died, the other 3 recovered. The mortality stopped from the date of the inoculation.

Barnyard 5. The epizootic began about November 25. By December 1, 7 of 60 chickens had succumbed. The 53 survivors were inoculated. Of these 4 were sick. The sick animals recovered and no new cases appeared.

Barnyard 6. The epizootic began on December 16. On the 28th, 40 of 142 fowls had died. The 102 survivors, of which 3 were infected, were inoculated. The sick recovered and the disease abruptly stopped.

Barnyard 7. The epizootic began on January 2. By January 14, 15 of 50 animals had died. The 35 survivors were inoculated. No new cases developed from this time on.

Barnyard 8. The epizootic began on about January 15 with a daily mortality of 4 to 6 fowls. On January 21 the 121 survivors, including 5 which were sick, were inoculated. The sick recovered and the epizootic stopped at once.

Barnyard 9. The epizootic began on about February 10. By February 20, 14 chickens had died from among the original 84. The 70 survivors were inoculated and the disease disappeared at once.

Barnyard 10. The epizootic began about February 25. By March 1, 20 chickens had died. The 120 survivors, of which 5 were sick, were inoculated. The 5 recovered and the epizootic stopped.

Barnyard 11. The epizootic began on February 4. From February 4 to 10, 10 chickens died. On February 10 the 48 living fowls were inoculated in the wing with 0.5 cc. of the antigallinarum bacteriophage, as had been all the chickens in the ten preceding experiments. The epizootic continued its course and 5 chickens died from February 10 to 17. On February 17 the 43 fowls which remained were inoculated with 0.5 cc. of a mixture of four strains of bacteriophage: active against *B. gallinarum*, *B. pullorum A*, *B. pullorum B*, and *B. pfaffi*. The epizootic stopped immediately after this second inoculation.

Barnyard 12. This barnyard was adjacent to the preceding and here the same facts were observed. A first inoculation made on February 9 on 80 chickens with a culture of the antigallinarum bacteriophage was without

effect. The epizootic stopped abruptly after an inoculation of bacteriophage active for the bacillary agents of the paratyphoses, made on February 17.

Examination of the blood of fowls dead in Barnyard No. 12 resulted in the isolation of a *B. pfaffi* type of bacillus. This organism, then, was responsible for the epizootics in groups 11 and 12. In this connection I will only mention the instance of the epizootic of paratyphosis at Trainel of which I have spoken in the chapter on avian typhosis. This outbreak was likewise due to *B. pfaffi* and was controlled by the inoculation of an anti-pfaffi bacteriophage.

Experiment II. This was performed at Poully en Auxois with the assistance of MM. Voillot and Bouhier, D.V.M.

Barnyard 1. On January 5, 20 chickens were taken at random from a poultry-yard containing about 100 fowls where typhosis had appeared. These 20 were immunized with a culture of anti-gallinarum bacteriophage. On February 7 the immunized birds were all alive and in perfect condition, while the epizootic had continued to spread among the non-immunized animals, of which only about 20 remained.

Barnyard 2. On February 23 the surviving chickens of a poultry-yard containing at that time 102 animals were immunized. The epizootic which began about 10 days previously, and which had resulted in a daily mortality of 4 or 5 chickens, stopped quickly and permanently from the time of the immunization. The epizootic continued, on the contrary, to ravage with the same intensity as formerly in all the neighboring poultry-yards which served as controls.

Experiment III. This experiment was conducted at Provins with the aid of M. Sorriau D.V.M., in an important poultry-yard where typhosis was present in endemic form.

For several months the daily mortality had been 2 or 3 fowls. On January 25 the 225 survivors were immunized. The epizootic immediately and permanently disappeared from the date of the immunization.

Experiment IV. Performed at Rouillac, Charente, with the assistance of M. Chollet, D.V.M.

On December 15, 100 fowls were immunized in a poultry-yard where typhosis had appeared about ten days previously. The daily mortality had been from 4 to 6 animals. With the immunization there was an immediate and permanent cessation of the epizootic. Typhosis continued to prevail on all the neighboring farms. Among the 100 chickens inoculated, about 12 were already affected. Of these only 2 died, 2 and 3 hours after the injection.

Experiment V. This test was conducted with the assistance of Dr. Ormières at Carcassonne.

The epizootic began during the month of August. By October 1, 80 chickens had succumbed. The 120 survivors were immunized. The epizootic stopped immediately and no further cases appeared after the date of the immunization.

Experiment VI. This experiment was conducted with the assistance of M. Mesnard, Departmental Veterinarian at Angoulême. In these experiments the chickens were immunized by the ingestion, on bread, of about 1 cc. of an antigallinarum bacteriophage.

A. On July 2 the 50 chickens surviving in a poultry-yard where typhosis had been prevalent for six weeks, with a daily mortality of 2 or 3 fowls, each ingested about 1 cc. of the bacteriophage culture. Seven months later no new case had developed since the time of the ingestion.

B. The same test was performed on October 15 on about 100 chickens on a neighboring farm where typhosis had been present fro several months. The epizootic was immediately and completely checked.

In both of these cases the disease continued to spread throughout the neighboring poultry-yards that were held as controls.

It appears needless to multiply such examples. In all cases the picture has been the same. The epizootic disappeared from the time that the culture of bacteriophage virulent for the pathogenic bacterium, the cause of the epizootic, had been introduced into the organism of the susceptible animal, whether this was brought about by injection or ingestion. We will see later that this last mode of administration is somewhat less efficient than injection.

On the contrary, injections of cultures of a bacteriophage active for *B. gallinarum*, the specific cause of typhosis, had in general, no effect when the epizootic was a paratyphosis, particularly in the case of infections due to *B. pfaffii*. In practise, it is only necessary to inject a mixture of different strains of bacteriophage active for the various pathogenic bacteria that may produce the epizootic. This mixture should also include a strain active for chicken cholera. It will be very easy to accomplish this, for the dose of 0.5 cc. which I have arbitrarily adopted

is indeed much larger than necessary, as we will see. Even in mixing five or six different strains of bacteriophage, the quantity necessary to effect immunization is not more than a fraction of a cubic centimeter.

In the course of the experiments cited there has been no selection. All of the animals of the poultry-yard, even though they were moribund, received the immunizing injection. About 100 sick chickens have therefore been injected, and the mortality among these has been 5 per cent. This is an appreciable reduction since the mortality among affected animals varies from one hundred per cent at the beginning of the epizootic to 95 per cent, when, after some weeks, the disease appears in only sporadic cases.

A culture of the bacteriophage, as we have shown in several ways, is composed of bacteriophagous ultramicrobes suspended in a medium containing the dissolved bacterial substance; the bacteria which have been destroyed by the action of the lysins secreted by the ultramicrobe. What, among these different principles, is the one which plays the active rôle in the protection of the healthy animal or in the one already sick, under the conditions of the experiment, that is, in a contaminated area? Unquestionably it is the bacteriophagous germs themselves. The *immediate* protection assured by the injection or even by the ingestion of the bacteriophage culture suffices to demonstrate this. An organic immunity necessarily requires a certain time for its development. Other phenomena of immunity, organic in nature, are produced only after an incubation period, as the experiments on barbone will show.

For the moment, let us conclude only that with sensitive animals immunized by the injection of a culture of the bacteriophage active for the causative pathogenic bacterium, in a *contaminated area*, that is to say, in an area where frequent reinfections may take place as a result of the dissemination of the pathogenic bacteria in the external environment, the principal rôle of protection is played by the bacteriophage itself. The other phenomena of immunity which may later develop, stimulated by the other substances contained in the cultures injected, play no rôle under such conditions, unless it be a very secondary

rôle. We will see that this proposition becomes reversed when similar experiments are carried out in a *non-contaminated area.*

IMMUNIZATION AGAINST BARBONE[2]

Thanks to the liberality of the Government of Cochin-China, which placed at our disposal all the animals, steers and buffaloes, which we needed, we have been able to study in detail certain of the conditions underlying immunization by means of the bacteriophage. Barbone is, indeed, an ideal disease for a study of this type. The blood taken from an animal about to die of the disease can be preserved in sealed ampoules for at least six months without any loss in the virulence of the bacteria present. Bouillon inoculated with a drop of this blood yields a culture which regularly kills the steer or the buffalo in a dose of 0.0002 cc. With half this dose, 0.0001 cc., usually one out of two animals will be killed. Experimental infection reproduces the spontaneous disease in the most minute details; the same temperature curve, the same symptoms, the characteristic edema at the point of entrance of the virus. Like the natural infection, the disease is fatal; all animals succumb and death occurs in the same length of time in the two cases, within twelve to eighteen hours of the appearance of the first symptoms. The lesions to be found at autopsy are identical. Immunization experiments conducted with such a disease provide, then, absolute results.

I may state here, once for all, that each time that the immunity of one animal has been tested by the inoculation of a culture of the bacterium of barbone this test has been controlled by the injection of an equal dose into a control animal of the same weight, and never has the control resisted. Furthermore, although there can be no possible doubt concerning the cause of death, confirmation has always been made by microscopic examination, by blood culture, and by the demonstration of the lesions at autopsy. The temperature of the experimental animals was taken regularly, morning and evening, and the slightest reaction in the immunized animals could not have passed unobserved.

[2] These experiments were conducted at Saïgon with the assistance of G. Le Louet, Chief of the Veterinary Service in Cochin-China.

The strain of bacteriophage employed for the preparation of the cultures destined for use in the immunization experiments had been isolated from the feces of a buffalo which had passed unaffected through the epizootic mentioned in the preceding chapter. This bacteriophage possessed, when derived from the organism, a strong virulence (+++) for the bacterium of barbone. After about ten passages *in vitro* the virulence became extreme (++++), and at this time it was used.

A fairly turbid bouillon culture of the bacterium of barbone about 12 hours old received one drop of the previously described active (++++) culture of anti-barbone bacteriophage. After about 12 hours the medium became perfectly limpid. This culture was filtered through a Chamberland filter (L_3) and distributed into ampoules, which were sealed. I would call attention to the necessity of employing only cultures with which the lysis of the bacteria has been complete. Such cultures ought, moreover, to be filtered because of the fact that a secondary culture may develop in some of the tubes.

The cultures of anti-barbone bacteriophage have been used after a variable length of time,—from twenty days to five months after their preparation. No difference has ever been observed in their mode of action, whatever the time elapsed between the date of preparation and the time of use.

All of the experiments, except those dealing with the effect of the age of the animal upon the development of immunity, have been effected on steers of the indigenous race, in a perfect state of health, aged from twelve to eighteen months, and of an average weight of 100 kgms.,[3] and on buffaloes aged from one to twelve years. The bovine race and the buffalo are equally susceptible to barbone. In Egypt, Piot has seen the herds of cattle decimated to the same extent as the herds of buffalo. According to our observations the buffalo may be rather easier to immunize than cattle.

Let us consider first the experiments conducted for the purpose of determining what conditions control the development of the immunity resulting from the injection of a culture of the bacterio-

[3] The race in Indo-China is of small size.

phage. The size of the dose and the age of the animals are the two principal factors whose variation has the greatest influence on the result. To facilitate discussion, we may consider the effects of smaller and smaller doses, although in reality the chronological order of the experiments was somewhat different, since the tests were first made with the injection of a dose arbitrarily fixed at five cubic centimeters. In this experiment the animals all died, when the test injection was given twenty days later. Thinking that the immunizing dose was inadequate it was increased in the next test to twenty cubic centimeters. Here again, the results were the same. It was only somewhat later, when smaller doses were employed, that the treatment proved to be efficacious. We have seen already that immunization by means of bacteriophage cultures presents individual peculiarities.

Determination of the immunizing dose

I. Eight steers received 20 cc. of the bacteriophage culture subcutaneously. Six of these were tested after a lapse of time varying from fifteen to forty days by the inoculation of a quantity of barbone culture representing certainly 50 fatal doses. All died in the same length of time as the control animals. The remaining 2 were tested, also with 50 fatal doses, sixty days after the immunizing injection. They showed no obvious disturbance. The two controls died in nineteen and twenty-two hours after the inoculation of virulent material.

II. Four steers received, subcutaneously, 5 cc. of the bacteriophage culture. Three were tested after thirteen, fifteen and twenty-eight days by the inoculation of 50 lethal doses of virulent bacilli. All died in the same length of time as the controls. The fourth was tested on the fortieth day. It showed no reaction. The control died in twenty-two hours.

III. Forty-one animals; 25 steers, 4 buffaloes aged from one to two years, and 12 adult buffaloes, received an injection of 0.25 cc. of the bacteriophage culture.

A. Eight steers were tested between the third and twelfth days following the injection by the inoculation of virulent culture, representing, according to the weight of the animal, from 5 to 1000 surely fatal doses. All died.

B. Twelve steers and one buffalo were tested between the 13th and 20th days, all by the inoculation of 1000 surely fatal doses of barbone culture. Five resisted, the others succumbed. The experiment is given in detail:

ANIMAL	TEST INJECTION 1000 FATAL DOSES GIVEN AFTER	RESULT
	days	
Buffalo	13	Resisted without showing any reaction
Steer no. 50	15	Died 26 hours after the inoculation
Steer no. 52	15	Died 20 hours after the inoculation
Steer no. 53	15	Died 23 hours after the inoculation
Steer no. 55	15	Died 26 hours after the inoculation
Steer no. 56	15	Died 25 hours after the inoculation
Steer no. 38	15	Resisted, without showing any symptoms
Steer no. 27	16	Died 68 hours after the inoculation
Steer no. 20	16	Resisted, without showing any symptoms
Steer no. 28	17	Resisted, without showing any symptoms
Steer no. 30	17	Resisted, without showing any symptoms
Steer no. 89	17	Died 34 hours after the inoculation
Steer no. 90	17	Died 32 hours after the inoculation

Two control steers died in 22 and 26 hours after the inoculation, and one buffalo, as control, died in 19 hours.

C. Twenty animals; 5 steers, 3 young buffaloes, and 12 adult buffaloes were tested during the period from the twenty-first to the sixtieth day after the immunizing injection. All received 1000 surely fatal doses of culture. *All* resisted without showing any reaction. Five control animals died, all between 16 and 23 hours after the inoculation of culture.

IV. Eight steers received 0.04 cc. of bacteriophage culture. They were tested after a variable number of days by the inoculation of 5 surely fatal doses of virulent culture. The results were:

ANIMAL	TESTED AFTER	RESULT
	days	
Steer no. 107	1	Resisted, no reaction whatever
Steer no. 103	1	Resisted, no reaction
Steer no. 106	2	Died 36 hours after the inoculation
Steer no. 101	3	Died 28 hours after the inoculation
Steer no. 83	4	Resisted, no reaction
Steer no. 84	4	Resisted, no reaction
Steer no. 104	5	Resisted, no reaction

The last steer, No. 102, was tested 60 days after the injection of the immunizing dose by the inoculation of 50 surely fatal doses of culture. It resisted without showing any disturbance.

V. A last experiment, as a control, was performed with a view to testing the practical application of immunization of buffaloes against barbone by M. Le Louet after my departure from Saïgon. Twelve steers received by

subcutaneous injection 0.25 cc. of bacteriophage culture. They were tested 25 days later by the inoculation of 2000 surely fatal doses of barbone culture. They resisted without showing the slightest reaction. The controls died in from 18 to 22 hours after the inoculation.

The injection of the bacteriophage did not produce in any of the animals, even in twenty cubic centimeter doses, the slightest reaction, either local or general. The temperature curve following the immunizing injection could be superimposed throughout on the curves of normal untreated animals. From this it is clear that, contrary to general belief, an immunity bordering on the refractory state may be acquired without the manifestation of the slightest reaction.

During the course of these experiments aphthous fever made its appearance at Saïgon. The animals in the course of immunization contracted it but this complication in no instance exerted any influence upon the development of immunity to barbone.

From these different experiments it may be deduced that with a large dose of bacteriophage culture the immunity is slow in being established; about forty to sixty days with a dose of twenty cubic centimeters, more than twenty-eight days with 5 cc. With 0.25 cc. it is not effective for all animals until about the twentieth day. It then permits them to resist without apparent discomfort two thousand surely fatal doses of the culture of barbone, that is to say, the immunity conferred borders on the refractory state. With the minimal dose of 0.04 cc., or less than a normal drop, a solid immunity is acquired by the fourth day. We are not concerned for the moment with the steers which have resisted after twenty-four hours; the immunity which they enjoy is of a different order, as we will see later.

We have seen in Part I of this text that the serum of rabbits which have received four injections of bacteriophage culture possesses the property of sensitizing the animals against the bacteria for which the bacteriophage injected was active. The delay in the establishment of the immunity as a result of the injection of large doses of antibarbone bacteriophage culture ought to induce this same phenomenon. The injection produces in the animals two phenomena of different orders: an immunity and a sensitization which varies in intensity according to the

dose inoculated. With a small dose the first surpasses the second which disappears quickly; with a large dose, on the contrary, the inhibitive action persists for a very long time—about sixty days for an injection of 20 cc. As we have seen in the rabbit the sensitization dominates and persists if the injections of the bacteriophage are repeated.[4]

The experiments further show that the immunity conferred by the injection of cultures of the bacteriophage is absolute when once established, and is negative during the period of incubation. There is no intermediary state. The animals, young or old, which receive the test inoculation during the period of incubation die, with very few exceptions, in the same time as the controls, even if this inoculation is made at a time very close to that where all the immunized animals resist. On the other hand, all those which are tested after the incubation period resist without presenting any apparent malaise, whatever the test dose may be. It seems indeed, as a result of these findings, that after an incubation time, more or less protracted according to the amount of bacteriophage culture injected, a period during which the animal remains as sensitive as a normal animal, the immunity increases very rapidly once its manifestation has commenced. In a word, the release of immunity is abrupt.

Effect of the age of the animals on the acquisition of immunity

We have seen that thirty-two animals, steers, young buffaloes, or adult buffaloes of less than twelve years, have all acquired an immunity that approaches the refractory condition within twenty hours of the injection of 0.25 cc. of the bacteriophage culture. We wished to see how this would compare with the results obtained in old animals.

Three buffaloes between fourteen and sixteen years and five very old animals no longer working and certainly more than

[4] Anaphylaxis shows a phenomenon of the same order. The smaller the sensitizing dose, the shorter the period of time before the animal is sensitized. For example, with a dose of 0.001 cc. of serum the guinea pig is sensitized after about fourteen days, with 5 cc. sensitization is present only after several months.

twenty years old[5] received 0.25 cc. of the culture of bacteriophage. All eight were tested forty-three days later by the inoculation of 1000 surely fatal doses of bacterium barbone culture at the same time as a normal control animal. This last died in seventeen hours. One of the three youngest buffaloes showed no reaction other than a transitory edema at the site of the inoculation, the other two showed a voluminous edema and were obviously sick, but all three recovered and could be considered normal six days after the test inoculation. The five very old buffaloes succumbed after 48, 53, 54, 60, and 142 hours; that is, after a time considerably longer than the control. Fifteen young animals immunized and tested at the same time failed to show any reaction to the test injection.

It is evident that although the test dose was enormous, that did not alter the fact that in the old animals the acquisition of immunity was much more difficult, somewhat in proportion to the age. The relative immunity against an extremely severe experimental test is observed only in these old animals; with the young or with adults in the prime of life, the immunity, as we have seen, is absent during the incubation period and complete once it has appeared at all.

The duration of the immunity

After my departure from Indo-China, my collaborator M. Le Louet, continued the experiments with a view to ascertaining the duration of the immunity produced by the inoculation of a culture of the bacteriophage. In January, 1921, he injected 15 steers, aged about one year, with a cubic centimeter of a culture of the bacteriophage that was about one month old, that is, a bouillon culture of the bacterium of barbone which had been

[5] The buffalo usually lives about twenty-five or thirty years. The Annamite never kills a buffalo; old and no longer able to work, it is fed and cared for as well as are the younger animals. The attachment of the natives for these buffaloes is such that it is difficult to find a person who will sell one of these animals. Those which served in the experiments were procured, some through the agency of the Governor of Cochin-China, M. le Gallen; others by M. Privé, Director of the plantations of An Loc and Suzannah, without considering the possible loss. I offer them my sincere thanks.

lysed by the bacteriophage one month before use. In March, 1922, all of the animals were tested, along with nine controls, by the inoculation of 0.1 cc. of a virulent culture of the bacterium of barbone. The virulence of this culture was such that in amounts of 0.002 cc. it regularly killed steers in less than thirty-six hours. Of the animals thus infected all of the controls died in from seventeen to twenty-three hours after the injection, while of the vaccinated animals ten resisted without any evident reaction and five died in from two to five days after inoculation.

This experiment shows that fourteen months after vaccination two-thirds of the animals possessed an immunity sufficiently strong to enable them to withstand a massive dose of the pathogenic bacterium.

The immunizing principle

Under the conditions of the experiment, that is to say, in a *non-contaminated area*, what, in the culture of the bacteriophage, is the principle which brings about the immunization?

A culture of the bacteriophage contains, as we know:

1. The bacteriophagous ultramicrobes, and

2. The soluble substances contained in the culture medium. These are the soluble substances derived from the bacterial bodies at the expense of which the bacteriophage has developed, the lysins secreted by these ultramicrobes and which remain in the medium once lysis has ended, and finally, somewhat later, the anti-lysins of defense secreted by the bacteria.

The course of the phenomenon alone, has shown us already that the immunizing principle must be different according as the immunity is developed in a *contaminated area*, as was the case in the experiments made on typhosis, or in a *non-contaminated area*, as in those on barbone. In the first, the immunity is acquired immediately; in the second, it becomes effective only after an incubation period. However, direct experiment allows us to confirm this idea.

1. If one injects steers, by the subcutaneous route, with from 5 to 20 cc. of anti-barbone bacteriophage culture it is possible to isolate the active ultramicrobe from the blood throughout the first twenty-four hours after the injection. After this period

they have disappeared. Experiment further shows that the ultramicrobes pass quickly into the intestine. They can be isolated from the intestine within about twelve hours after the injection and they persist there for a somewhat longer time than in the circulation: for two or three days (up to six days in a single case). In all instances they have disappeared long before the immunity is established. Let us repeat that this applies only to the case where the introduction of the bacteriophage into the organism takes place in a territory free from the infection. We have seen, for example, that five months after the termination of an epizootic of barbone it is still possible to isolate a bacteriophage active for the pathogenic bacterium from the excreta of buffaloes which have resisted. On the other hand experimentation in the chicken has shown us that the activity of the bacteriophage for the pathogenic bacillus is maintained just as long as the experimental animal continues to ingest these bacteria.

2. Bablet has shown that the bacteriophagous germs are destroyed by preservation for a week in glycerine. We know that this substance exerts no destructive influence on either the diastases or the toxins. It may be assumed, therefore, that in a mixture of bacteriophage culture and glycerine the ultramicrobes alone will be destroyed while the immunizing substances contained in the medium will remain intact. Starting from this hypothesis, we mixed 0.5 cc. of a culture of the anti-barbone bacteriophage with 9.5 cc. of glycerine. After holding the mixture at incubator temperature (37°C) for ten days, and after we were assured that the bacteriophagous ultramicrobes were effectively destroyed, we inoculated two steers with this liquid, diluted in 500 cc. of saline. Each steer received then 0.25 cc. of the original culture. Tests after forty-five days, respectively with 5 and 50 fatal doses of a culture of the bacterium of barbone, showed that these two animals resisted. They had acquired an immunity in spite of the destruction of the bacteriophagous ultramicrobes.

In the case of experimental barbone the tests were made in a barbone-free region, and the principle which is responsible for the development of the immunity is most probably constituted of the substance of the bacterial cells. The rôle which the bacteriophage plays here is to dissolve the bacteria, in which condi-

tion the bacterial substance is in a state particularly adapted to stimulating the cells of the body which enter into the production of organic immunity. The substance of the bacterial body dissolves in the medium under the influence of the lysins secreted by the ultramicrobes, but it is not present in the same condition as in the body of the living bacterium, for the bacteriophage does not simply produce a disintegration. This is shown by the fact that the culture medium becomes perfectly limpid, whereas the medium remains cloudy when a simple disintegration takes place. As we have seen in several tests, the destruction of the bacterium by the activities of the lysins—the diastases—is a process of solution. Indeed, it is rather the substances composing the bacterial body which are dissolved. This process is of necessity accompanied by a change in state. It is, then, not proper to speak of the bacterial substance as the principle which provokes the acquisition of immunity; it is in reality the products resulting from the degradation, under the influences of lysins secreted by the ultramicrobes, of the substances composing the bacterial cells which are effective.

It is obvious that this is yet only an hypothesis, experiment showing only that the principle which provokes the appearance of immunity is not, under the conditions of the experiment, the bacteriophage considered as a living being. Aside from the dissolved bacterial substance do the diverse principles present in the culture, namely, the bodies of dead ultramicrobes, the lysins, and eventually the anti-lysins, play any part in the production of immunity? In the present state of these investigations it is impossible to affirm or deny this.

We have tested the action of temperature on the immunizing element contained in the bacteriolysate. To this end, we have repeated the experiment of the culture of the bacteriophage treated with glycerine, with the difference that the culture has previously been subjected to a temperature of 56°C. maintained for a half hour. Two steers have each received a dose of this culture, heated and glycerinized, corresponding to 0.25 c.c. of the original culture. After forty-five days they were tested, the one with five, the other with fifty, fatal doses of barbone culture. The first resisted, the second died. The immunizing principle contained in the

bacteriophage culture is not destroyed but is sensibly weakened by heating for a half hour at 56°C.

Although it is not yet possible to know with certainty the nature of the process which controls the development of organic immunity, we are at least able to recognize the result and to note the property which distinguishes the animal immunized by an injection of the bacteriophage from a normal animal.

In the case of the immunity acquired as a result of an attack of a contagious disease the blood possesses preventive properties. The blood of immunized animals enjoys the same property, as the following experiments show.

I. Steer no. 54 received on November 5, 0.25 cc. of an anti-barbone bacteriophage culture. Fourteen days later 500 cc. of blood was taken into a flask containing 25 cc. of a 10 per cent solution of sodium citrate. The blood was immediately injected into the jugular vein of steer no. 43. This last animal was tested twenty-three hours later by the injection of 1000 fatal doses of the bacterium of barbone culture. It failed to show the least evidence of infection. A control died in twenty-three hours. Steer no. 54 likewise resisted the inoculation of 1000 fatal doses, given on December 1st.

II. The experiment given above was repeated. Steer no. 112 received into the jugular vein 500 cc. of blood from steer no. 95. Both of them resisted the test injections.

III. Steer no. 104 received on December 29 a subcutaneous injection of 0.04 cc. of a culture of the bacteriophage. Four days later 500 cc. of blood were taken, as before, and this was transfused into steer no. 108. The next day the two steers resisted the inoculation of five fatal doses, which killed the control animal in thirty-two hours.

IV. The above experiment (III) was repeated. The steer which received the blood of the immunized animal was not tested by the inoculation of 50 fatal doses until forty-five days after the transfusion. It resisted, without showing any apparent disturbance, as did also the steer which was immunized directly.

This last experiment does not, however, prove anything with regard to the duration of passive immunity conferred by the blood of an immunized animal, for it was performed with homologous blood, and we know that an immunity thus produced is of much longer duration than when produced with heterologous blood. In all cases, the immunity thus conferred is extremely powerful and these experiments open the way for further investigations

on the production of therapeutic sera in animals immunized by a *single* injection of an active bacteriophage, not only for barbone, but for other diseases as well.

One might conceive that the "principle" which is contained in the blood of the immunized animal and which confers the passive immunity does not differ from the culture of the bacteriophage which persists for a certain length of time in the circulation. But this is impossible, for if the blood is taken at a time sufficiently close to the immunizing injection of the bacteriophage it is in no way effective. That is, blood taken during the incubation period confers no immunity to the transfused animal.

Steers nos. 89 and 90 received on December 19, 0.25 cc. of the bacteriophage culture subcutaneously. Sixteen days later 500 cc. of blood were withdrawn from each animal and transfused into steers nos. 92 and 93. The four animals, tested the next day, died with no greater delay than the controls. Steer no. 46 received on November 5, 20 cc. of the culture of bacteriophage. On November 19, 500 cc. of blood were taken and transfused into steer no. 42. These two animals died in the same time as the control after a test injection.

As is to be seen, the incubation period of immunity in animals which receive the immunizing injection of bacteriophage culture parallels the appearance of the protective power in their blood. Immunity develops abruptly; in the same way the protective power of the blood manifests itself suddenly, and at the same moment.

What then, is the immunizing principle which makes its sudden appearance in the blood at the moment when immunity is established, even in animals which have received only the minimal dose of a single drop of the culture of the bacteriophage? Can it be an amboceptor? By no means, for the complement fixation reaction shows that the sera of animals immunized with cultures of the bacteriophage do not contain a specific amboceptor in detectable quantity. In conducting the reaction of Bordet with even 0.5 cc. one obtains an exactly comparable hemolysis, of the same intensity and in the same time, as that which occurs in a control tube containing the same quantity of normal serum.

Examination of the opsonic power of two of these sera gave indices of 0.3 and 0.4; indices which are essentially negative.[6]

The serum containing the protective principle does not contain inhibiting substances or even substances delaying the growth of the bacterium of barbone. Bouillon, mixed with such a serum, in any proportion (from 0.05 cc. to 3 cc. per 10 cc. of bouillon), with or without the addition of fresh guinea pig serum, furnishes a medium which, when inoculated, gives luxuriant cultures of the bacterium of barbone.

Finally, the serum contains no traces of agglutinins.

Organic immunity, then, is not due to the presence of an amboceptor, nor to the presence of an opsonin in the blood of the vaccinated subjects. The blood contains neither agglutinins nor inhibiting substances. The immunity is most probably antitoxic.

We have seen in the experiments performed on avian typhosis, that, in an *infected area*, the protection of the animal is immediate and that this protection is assured only by the presence of bacteriophagous ultramicrobes virulent for the pathogenic bacterium. We have again found this immediate immunity in the case of barbone. It is that which protected steers nos. 103 and 107 against the inoculation of five fatal doses of culture when given only twenty-four hours after the injection of the bacteriophage.

In typhosis, this heterologous immunity has been permanent, for the daily reinfections which occur in the infected area allow the bacteriophage to multiply at the expense of the pathogenic bacteria ingested and thus to maintain its virulence for this bacterium. In barbone, this same thing takes place in an infected area, since we have seen that the bacteriophage virulent for the bacterium of barbone was present in the intestine of buffaloes five months after the complete disappearance of the epizootic.

[6] We have seen in Part I that the lysin secreted by the bacteriophage possesses a very high opsonic power. The organism must respond to an injection of lysin (certainly present in the culture of the bacteriophage) by the production of an anti-opsonic antibody.

In a non-infected region, and this was the case in the experiments performed on barbone, the mechanism is not the same. In the absence of reinfection the bacteriophage active for the bacterium is eliminated very rapidly from the organism, since it is not able to multiply at the expense of this bacterium. The heterologous immunity disappears with it,—that is to say, after one or two days,—and the animal then becomes susceptible. It remains in this condition throughout the entire duration of the incubation of the organic immunity, which develops under the influence of the soluble products contained in the culture of the bacteriophage. Once this organic immunity is established the animal is refractory.

We will see in connection with dysentery, that when a culture of the bacteriophage is injected the organism responds by the production of an antitoxin. It is probable that the same thing takes place in barbone and that the protective principle present in the blood, since it is neither an amboceptor nor an opsonin, is likewise an antitoxin; the response of the organism to the injection of the modified substance of the lysed bacterial cells contained in the culture of the bacteriophage.

To summarize: the injection of the buffalo or of cattle, with a culture of bacteriophage active for the bacterium of barbone confers:

1. An heterologous immunity, solely due to the presence in the body of bacteriophagous ultramicrobes virulent for the bacterium of barbone, which assures the destruction of the bacteria upon their introduction into the organism. This immunity terminates just as soon as the ultramicrobes are eliminated from the body. In the absence of frequent reinfections this elimination is very rapid, since the continued growth and the maintenance of virulence can not persist.

2. An homologous immunity, or organic and powerful immunity, induced by a reaction of the organism of the animal to the soluble principles contained in the culture of bacteriophage injected. This organic immunity is characterized principally by the appearance in the blood of an extremely potent immunizing substance—probably an antitoxin. The organic immunity establishes itself abruptly after an incubation period, which varies with the dose injected, being longer as the amount of injected culture is increased.

A single injection of 0.04 cc., or less than a normal sized drop, into a steer of 100 kgms. weight places the animal within 4 days in a condition where it can withstand a test inoculation of five fatal doses. Sixty days later the animal resists a test inoculation representing fifty surely fatal doses.

The blood of an immunized animal injected into a normal animal confers on the latter a passive immunity as solid as that enjoyed by the actively immunized one itself, even if this last one has received but a single injection of 0.04 cc. of culture of the bacteriophage. And this passive immunity, under experimental conditions at least, is still intact forty-five days after the injection of the blood.

IMMUNIZATION AGAINST DYSENTERY

"The cultures of Shiga lysed by the invisible microbe, which are in reality cultures of the anti-microbe, possess the property of immunizing the rabbit against a dose of Shiga bacilli which will kill the controls in five days." This statement is taken from my first communication on the bacteriophage. The experimental data upon which this affirmation was based are given in the following protocols.

The rabbit, although naturally refractory to bacillary dysentery is, on the contrary, susceptible to the inoculation of dysentery toxin. This animal could, then, be utilized for the *preliminary* antitoxic immunization experiments. The following experiments showed at first that the culture of anti-Shiga bacteriophage, a short time after lysis, is toxic, although to a less degree than is a normal culture of Shiga bacilli.

Rabbit no. 1. One cubic centimeter of a normal culture of Shiga bacilli was injected intravenously on August 10. The animal died on August 16.

Rabbit no. 2. Two cubic centimeters of a normal culture of Shiga bacilli were injected subcutaneously on August 10. The animal died on August 16.

Rabbit no. 3. One cubic centimeter of a Shiga bacillus culture which had been subjected to lysis for six hours was injected intravenously. (The amount of bacillary substance here was the same as in the preceding.) The rabbit lived.

Rabbit no. 4. Two cubic centimeters of a Shiga culture which had been lysed for six hours were injected subcutaneously. The animal died on August 16. This rabbit had also been injected on August 10.

Six days after the completion of the lysis the toxicity of the culture was markedly diminished, as the following tests show:

Rabbit no. 5. Two cubic centimeters of a Shiga bacillus culture which had been lysed for six days were injected intravenously on August 10. The animal lived.

Rabbit no. 6. Three cubic centimeters of the Shiga culture which had been lysed for six days were injected subcutaneously on August 10. This rabbit also lived.

Rabbit no. 7. Five cubic centimeters of the Shiga culture which had been lysed for six days were injected intravenously on August 10. The rabbit died on August 21.

When the tests were done with a Shiga culture which had been lysed for a month the toxicity had disappeared, as is shown by the following.

Rabbit no. 8. Fifteen cubic centimeters of Shiga culture which had been lysed for one month were injected subcutaneously. The rabbit lived. The injection was given on August 10.

Rabbit no. 9. Ten cubic centimeters of this same culture were injected intravenously on August 10. This rabbit lived.

The following protocol illustrates an immunization experiment.

On August 23, eight rabbits received a subcutaneous injection of 0.25 cc. of a culture of the anti-Shiga bacteriophage, two months after the lysis was completed. These animals were tested by the injection of 3 cc. of a twenty-four hour bouillon culture of Shiga bacilli. For the strain employed this represented two surely fatal doses. The strain of Shiga used in the test differed from that used to prepare the suspension lysed by the bacteriophage.

Rabbit no. 10; tested after twenty-eight hours. Died six days later.
Rabbit no. 11; tested after four days. Died five days later.
Rabbit no. 12; tested after six days. Lived.
Rabbit no. 13; tested after eight days. Lived.
Rabbit no. 14; tested after ten days. Lived.
Rabbit no. 15; tested after one month. Lived.
Rabbit no. 16; tested after two months. Lived.
Rabbit no. 17; tested after three months. Lived.

All the control rabbits inoculated with half the dose, that is, with 1.5 cc. of the Shiga culture, died in from four to seven days.

The rabbit is therefore, immunized against two surely fatal doses of *B. dysenteriae* Shiga culture by the injection of a quarter

of a cubic centimeter of a culture of the anti-Shiga bacteriophage. The antitoxic immunity is established six days after the injection and persists for at least three months.

In an experiment of this kind there can be no question of the nature of the process. The bacteriophage as a living being can not be the cause of the immunity. The responsible agent must be the soluble principles contained in the culture medium.[7]

Before undertaking experiments on man I had to assure myself that the administration of cultures of the anti-Shiga bacteriophage caused no reaction. First, I ingested increasing quantities of the cultures, aged from six days to a month, from one to thirty cubic centimeters, without detecting the slightest malaise. Three persons in my family next ingested variable quantities several times without showing the least disturbance. I then injected myself subcutaneously with one cubic centimeter of a forty-day old culture. There was neither a local nor a general reaction. In all the cases, twenty-four hours after the ingestion or after the injection, I was able to isolate from the stools a bacteriophage possessing for the Shiga bacillus an activity equal to that of the ultramicrobe administered. More recently G. Eliava has received by subcutaneous injection 5 cc. of a culture of the anti-Shiga bacteriophage aged thirty days. No reaction, local or general, followed.

It is known that the subcutaneous injection of Shiga bacilli, killed by any procedure whatsoever, can not be performed because of the extremely violent reactions produced, and which are due to the toxicity of the germ. This is precisely the reason that vaccine prophylaxis is not applied to dysentery as it is in the case of typhoid. The absolute innocuity of injections of

[7] Several immunizing experiments with the bacteriophage for *B. typhosus* and for the paratyphoid organisms have been performed upon laboratory animals, both rabbits and guinea pigs. In all cases these showed a perfect immunization;—provided it is permissible to employ the word immunization when the process is carried out in refractory animals.

Not attributing any value to experiments of this type I have not included them in the monograph. In all cases the bacteriophage administered, either when given by subcutaneous injection or by the buccal route, has been isolated a few hours later from the intestinal tract.

the anti-Shiga bacteriophage cultures, which contain the substance of the bacterial bodies in a dissolved state, shows indeed that these substances undergo profound modifications under the influence of the lysins secreted by the bacteriophagous ultramicrobe. Nevertheless, these new substances possess a specific immunizing power much more potent than the original substance. The experiments on rabbits, and in particular the results secured in immunization against barbone, demonstrate this beyond possible doubt.

Prophylactic vaccination against bacillary dysentery by means of cultures of the anti-dysentery bacteriophage is therefore applicable to man. In practice, quite naturally, the prophylactic injections should be performed with a mixture of bacteriophage cultures—anti-Shiga, anti-Flexner, and anti-Hiss. Such a mixture would constitute a polyvalent dysentery vaccine.

The Shiga bacillus is one of the most toxic organisms known, and it may be assumed that the harmlessness of injections of such a culture indicates a general law, whatever may be the bacterium against which the bacteriophage culture is prepared. In order to test this hypothesis, I injected myself, subcutaneously, with half a cubic centimeter of anti-plague bacteriophage. No reaction, either general or local, followed. Stool examination made twenty-four hours after the injection showed that the bacteriophage, equal in virulence to that injected, was present. The inoculation experiment was repeated with anti-typhoid bacteriophage. G. Eliava repeated it with the anti-staphylococcus bacteriophage, and the same results were secured in both cases. These observations are confirmed in part by another fact, observed in several tests, that following the administration of the bacteriophage, either by injection or by ingestion, the bacteriophage passes in a short time into the intestine. It is eliminated rapidly if it fails to encounter the bacterium against which it has a virulence, that is to say, in an uninfected individual. On the contrary, it grows and maintains its virulence if it is in contact with this bacterium, a condition which, as we have seen in several instances, is produced in an infected environment among animals which remained healthy, or which had been infected and were recovered.

After being assured of the innocuity of the ingestion of cultures of the anti-Shiga bacteriophage, this treatment was applied for therapeutic purposes to patients affected with bacillary dysentery.[8] As in the experimental work, so also here in the clinical tests, the therapy has been limited to those cases in which the etiology of the infection was proved by the isolation of the pathogenic organism, and where, in addition, the virulence of the intestinal bacteriophage was negative toward the different dysentery bacilli at the time of the administration of the culture of bacteriophage. It is evident that in routine practice it would not be necessary to investigate all these points, especially since the administration of the bacteriophage cultures is always inoffensive.

In each of the following cases the only treatment instituted has been the ingestion of the culture of the bacteriophage.

Robert K. . . . (eleven years). This is a case of bacillary dysentery of moderate severity with from 5 to 7 bloody stools a day.

August 1. The stool examination showed: *B. dysenteriae* Shiga present.

The intestinal bacteriophage with virulences as follows: B. *coli* ++, Shiga 0, Flexner 0, Hiss 0.

August 2. At 10 o'clock in the morning the patient ingested 2 cc. of a anti-Shiga bacteriophage culture. This culture had been lysed for thirty-five days. During the afternoon of this day there were 3 bloody stools, in the evening there was one stool and that was free of blood.

August 3. During this day there was only the one formed stool. Examination showed: *B. dysenteriae* Shiga absent.

The intestinal bacteriophage with virulences as follows: *B. coli* ++++, Shiga ++++, Flexner +++, Hiss +++.

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[8] These experiments have been made with the assistance of M. Nadal, on the service of Pr. Hutinel, at the Hôpital des Enfants Malades.

Tests have also been made in cases of toxic diarrhea of infants, but they will not be discussed here since a conclusion regarding them has not yet been reached. In those cases there is an especial difficulty, for the pathogenic organism is still unknown. It was at first thought that this might be determined through the ability to isolate and cultivate an active strain of bacteriophage which might be used for curative purposes. It is indeed probable that there is, not one, but several diarrheas of infants caused by different bacterial types, as the experiments of Nobécourt made during the past few years would also indicate. The solution of the problem is not impossible but it would be necessary to administer to the affected infants a mixture of cultures of diverse strains of the bacteriophage, active against the diverse bacterial types capable of inciting the diarrhea. It can readily be conceived that under such circumstances the investigation must be protracted.

August 8. The intestinal bacteriophage was active as follows: *B. coli* +++, Shiga +, Flexner 0, Hiss +.

August 9. The patient was discharged from the hospital.

André B. . . . (ten years). A case of bacillary dysentery of moderate severity. During the period from August 25 to 29 inclusive there were 9 to 11 bloody stools a day.

August 28. Stool examination showed: *B. dysenteriae* Shiga present.

Intestinal bacteriophage active as follows: *B. coli* +, Shiga 0, Flexner 0, Hiss 0.

August 29. At 4 p.m. the patient ingested 2 cc. of an anti-Shiga bacteriophage culture. The culture had been lysed for two months.

August 30. There was one bloody stool in the morning and during the afternoon and the night there were 5 stools, none of which showed any blood.

August 31. There were 3 fluid, but not bloody, stools.

Examination showed: Shiga bacilli not present.

The intestinal bacteriophage active as follows: *B. coli* ++++, Flexner ++, Hiss +++, Shiga ++++.

September 1. There was one fluid stool, without blood.

September 2. There was one fluid stool, without blood.

September 3. There was one formed stool. Examination of the intestinal bacteriophage showed: *B. coli* +++, Shiga ++++, Flexner +++, Hiss +.

September 8. Reactions with the intestinal bacteriophage were: *B. coli* ++, Shiga 0, Flexner 0, Hiss +.

September 9. The patient was discharged from the hospital.

Robert D. . . . (twelve years). This patient had a very severe dysentery, with vomiting, cold sweats, chilling of the extremities, and involuntary and uncountable stools.

September 8. The stools could not be counted. They were fetid, purulent, and streaked with blood. Examination showed: *B. dysenteriae.* Shiga present; about 1 out of every 10 colonies on the plates was the dysentery bacillus.

The intestinal bacteriophage showed no virulence for *B. coli,* or for the Shiga, Flexner or Hiss organisms.

September 9. Two cubic centimeters of an anti-Shiga bacteriophage culture were ingested at 11 o'clock. This culture had been lysed for three and one-half months. During the afternoon and the night the stools became less numerous but continued bloody.

September 10. There were 6 fluid stools, without blood. Examination showed: *B. dysenteriae* Shiga, not present.

Intestinal bacteriophage active as follows: *B. coli* ++++, Shiga ++++, Flexner ++++, Hiss +++.

September 11. There were 2 normal, formed stools.

September 20. The patient was discharged from the hospital.

Julien D. . . . (three and one-half years). This was a case of very severe dysentery. The general condition of the patient was very bad. A sister of the patient had died at home of dysentery on September 8.

From the 11th to the 13th of September the number of stools, all of which were bloody, could not be counted.

September 13. The patient entered the hospital. Examination showed: *B. dysenteriae* Shiga present, the dysentery bacilli constituting about 4 of every 5 colonies on the plates.

The intestinal bacteriophage was without activity for either *B. coli* or the dysentery organisms.

September 13. The patient ingested 2 cc. of anti-Shiga bacteriophage culture at 5 o'clock. This culture had been lysed for fifteen days.

September 14. There were 6 bloody stools. The intestinal bacteriophage showed virulences as follows: *B. coli* +++, Shiga +++, Flexner ++, Hiss +.

September 15. During the day there was one bloody stool. There were also 5 stools without blood. Examination showed: *B. dysenteriae* Shiga absent.

The intestinal bacteriophage with activities as follows: *B. coli* ++++, Shiga ++++, Flexner +++, Hiss +++.

September 16. There were 4 stools, all without blood. The intestinal bacteriophage showed: *B. coli* ++++, Shiga ++++, Flexner ++, Hiss +++.

September 17. During the day there were one fluid stool and 2 formed stools. The intestinal bacteriophage showed: *B. coli* ++++, Shiga ++++, Flexner ++, Hiss +.

September 18. There were 2 formed stools. The intestinal bacteriophage was virulent as follows: *B. coli* ++++, Shiga ++++, Flexner +, Hiss ++.

September 26. The patient was discharged from the hospital. On this date the virulence of the intestinal bacteriophage was: *B. coli* +++, Shiga 0, Flexner 0, Hiss+.

Emile D. . . . (seven and one-half years). This patient was a brother of the preceding case, and showed a very severe dysentery. On the 11th and 12th of September there were 20 to 25 fetid stools, fluid but not bloody.

September 12. Examination showed that the Shiga bacilli were very abundant. The intestinal bacteriophage was inactive for *B. coli* or for the dysentery organisms.

September 13. There were 25 bloody stools. At 5 o'clock the patient ingested 2 cc. of bacteriophage culture. The culture had been lysed for six and one-half months.

September 14. There were 4 bloody stools in the morning and 2 stools without blood in the afternoon. Examination of one of the latter showed no Shiga bacilli. The virulences of the intestinal bacteriophage were: *B. coli* ++++, Shiga ++++, Flexner ++, Hiss +++.

September 15. There were 5 stools, all free of blood.

September 16, 17, and 18. During these days there were 3 or 4 stools a day. None of these contained blood.

September 19. There were 2 formed stools on this day.

September 28. The patient was discharged from the hospital.

In all of these cases the general condition of the patient has always coincided with the severity of the intestinal symptoms.

Two other cases of dysentery due to the Shiga bacillus, treated in the same manner, but outside of the hospital, gave comparable results. In these there was a cessation of the bloody stools with improvement in the general condition in the twenty-four hours immediately following the administration of the culture of anti-Shiga bacteriophage.

Obviously, seven cases are not sufficient to afford an absolute proof in favor of the specific therapy of bacillary dysentery by means of bacteriophage cultures. However, they do suffice to show that the ingestion of cultures of a virulent bacteriophage—virulent for the infecting bacillus—is as harmless for the sick as for the healthy person. They also show that the ingested bacteriophage traverses the upper digestive tract in man as it does in animals, and within a few hours will be found in the intestine where it grows at the expense of the bacterium for which it is active. Moreover, these seven cases acquire a significance from the fact that the cultures of bacteriophage restrained the disease, as was the case in avian typhosis, as is shown in the results of the experiments which have been recorded and in experiments bearing on about one hundred cases.

All these facts authorize clinicians to continue the experimental treatment on a more elaborate scale, not only in bacillary dysentery but in other infectious human diseases for which strains of the bacteriophage have been isolated—typhoid fever, the paratyphoid infections, and bubonic plague.[9]

Whatever may be the nature of the disease under consideration, the isolation of a strain of the bacteriophage active for the pathogenic bacterium is easy once it appears in an acute disease

[9] It may also be suggested that the bacteriophage may have an application in surgery, as in the treatment of wounds or in peritonitis, either as a preventive when an infection is to be feared, or as a therapeutic agent when infection has once appeared.

where the bacterium is known and cultivable. It is only necessary to apply the principles which have been discussed in the course of this work.[10]

[10] Since the publication of the French edition of this work Bruynoghe and Maisin at the Bacteriologic Institute of Louvain, and Beckerich and Hauduroy at the Institute of Hygiene at Strasbourg have confirmed these conclusions.

Bruynoghe and Maisin have treated with success affections due to the staphylococcus and anthrax by the subcutaneous injection of cultures of the bacteriophage.

Beckerich and Hauduroy have experimented with typhoid fever and with pyelocystitis. They have employed cultures of the bacteriophage virulent for the causative bacterium, heated to 58°C. for thirty minutes. They record the following cases;

1. An adult with typhoid fever of moderate severity. The patient ingested 2 cc. of bacteriophage culture on the 18th day of the disease. Defervescence took place forty-eight hours later.

2. An adult with typhoid fever of moderate severity. The patient received the same treatment, with the same results. The culture was given on the ninth day of the disease.

3. An infant with severe typhoid fever. The patient was given 2 cc. of the bacteriophage culture by mouth and in addition the simultaneous injection of 1 cc. This treatment was given on the twentieth day. The temperature came down within forty-eight hours and the apyrexia was permanent.

4. An infant with a paratyphoid B infection, whose condition was grave. On the ninth day the bacteriophage was given by the simultaneous ingestion and injection of the culture. After the next day the apyrexia was permanent.

5. An infant with paratyphoid B infection of average severity. On the 23rd day the bacteriophage was given by ingestion and injection. The apyrexia was permanent after the next day.

Two adults affected with typhoid fever of the ataxo-adynamic form and with pronounced myocardial involvement were treated. The apyrexia which followed the treatment within forty-eight hours did not prevent death. Considering that in these two cases the failure might be due, either to a too delayed intervention, or to too small a dose in view of the severity of the cases, they decided to increase the quantity of bacteriophage culture administered by ingestion in such cases.

6. An infant affected with typhoid fever in very severe form. On the tenth day the patient was given 5 cc. of culture by mouth and a simultaneous injection of 1 cc. Within forty-eight hours there was permanent defervescence with euphoria.

7. An infant with a very severe case of typhoid fever. The same treatment was given on the fourteenth day. Defervescence with euphoria occurred in forty-eight hours.

These authors confirm the absolute harmlessness of the administration of cultures of the bacteriophage, whatever may be the mode of introduction into the organism. The sole reaction which they observed consisted of a sudoral crisis which followed the administration in about two hours, even when the administration was effected by the oral route. They consider that this reaction is due to the lysis of the pathogenic bacteria within the body of the patient. This view is certainly correct for this sudoral crisis is not observed in the healthy individual, either after the injection or after the ingestion of cultures of the bacteriophage. This has been demonstrated in several instances.

They note the constant coincidence which exists between the administration of the culture and the apyrexia which follows within forty-eight hours. This relationship appears to be independent of the stage of the disease when the culture is given. In all the treated cases blood cultures made forty-eight hours before the intervention were positive, that is, the treatment was always applied at a period when the disease was fully active.

Beckerich and Hauduroy have, moreover, treated two cases of puerperal pyelocystitis due to *B. coli* by the subcutaneous injection of 1 cc. of an anti-coli bacteriophage culture. In both cases the sudoral crisis followed in two hours, and permanent apyrexia in forty-eight hours.

CHAPTER IV

The Bacteriophage and Immunity

SUMMARY AND CONCLUSIONS

The bacteriophage, *Bacteriophagum intestinale* d'Herelle, 1918, an ultramicrobial parasite of bacteria, normally exists in the intestinal tracts of animals, both vertebrates and invertebrates. The possibility of counting the ultramicrobes is a most important point in the study of the bacteriophage, for it makes it possible to follow its development and to recognize its mode of action *in vitro* and *in vivo*. An obligatory parasite, the bacteriophage lives only at the expense of living, normal bacteria, which constitute its sole culture medium. Experiments and ultramicroscopic examination agree in showing that the ultramicrobial bacteriophage penetrates into the interior of the bacterium, there forms a colony of fifteen to twenty-five elements within one to one and one-half hours; whereupon the bacterium bursts and liberates the young ultramicrobes. For its development the bacteriophage utilizes the substance of the bacteria which it dissolves by the aid of the lytic diastases which it secretes. The property possessed by the bacteriophage of secreting a lysin, active for a given bacterium,—that which permits it to penetrate this bacterium and to reproduce there—represents, in the strict sense of the word, its virulence for this bacterium.

There is but a single species of bacteriophage, common to all animals, capable of acquiring virulence for different bacterial species, probably for all species.

Just as for each pathogenic bacterium there is a scale of virulence for a given animal, so also for each bacteriophage there is an individual virulence. We are able to increase or attenuate the virulence of the pathogenic bacterium, and the same phenomenon can be obtained with the bacteriophage. The parasitized superior organism defends itself against the bacterial secretions and is able to acquire an antitoxic immunity; the bacterium at-

tacked by the bacteriophage does not remain passive. It resists, and is able to overcome the ultramicrobe and to acquire an antilytic immunity. All the vicissitudes of the struggle between the animal and the bacterium have their counterpart in the struggle between the parasitizing bacteriophage and the bacterium attacked. The resemblance is complete. It is simply a matter of descending a degree in the order of size of the beings involved.

The existence of the bacteriophage in the intestine of all living beings, its exiguity which allows it to filter through soils impermeable to bacteria, its vitality and resistance to agents of destruction, explain its extreme diffusion in nature.

When derived from the organism a single strain of the bacteriophage is rarely active for but a single bacterial species. Usually it attacks several species at one and the same time, and for each it possesses a separate and variable virulence. There is but one bacteriophage but there is an infinity of strains, each possessing, when taken from the organism, the power of attacking a certain number of bacteria. A single strain is variable from time to time, both as to its intensity of action against each bacterial species, and as to the extent of its action with regard to the number of bacterial species attacked. All combinations of virulence being possible in quantity as in quality, it can be understood in view of the infinite number of possible combinations, that there can exist no two strains of ultramicrobial bacteriophage which can be exactly alike.

In a bacterial suspension inoculated with a culture of an active strain of bacteriophage it is not always the latter which prevails. If the bacterium succeeds in acquiring a resistance a selection of more and more resistant bacteria occurs, resulting in the formation of a state of equilibrium between the resistant bacterium and the virulent bacteriophage which then coexist in the medium. A mixed culture results in which the equilibrium is more or less stable but which can be overthrown in favor of the one or the other of the germs there present, according to the circumstances of the moment.

The acquisition of resistance is accompanied by changes in morphology and in the properties of the bacteria; the bacilli take a coccoid aspect and become surrounded by a capsule.

They become inagglutinable. They resist phagocytosis. They are endowed with a very great vitality and a very high virulence. Loss in resistance is accompanied by a return to normal form and properties.

Although the bacteriophage is capable of acquiring a virulence for the bacterium, the bacterium on its side is capable of acquiring a resistance to the bacteriophage. The virulence of the one and the resistance of the other are not fixed, but are essentially variables, being enhanced or attenuated according to the inherited properties of each of the two germs, and according to the circumstances of the moment which favor the one or the other of the two antagonists. These two phenomena dominate the pathogenesis and the pathology of infectious diseases.

The bacteriophagous ultramicrobe is a normal inhabitant of the intestine, an obligatory parasite, and there maintains itself at the expense of saprophytic bacteria hereditarily endowed with a certain resistance, with which it lives in commensalism. For any bacterium whatever, pathogenic or not when introduced into the intestine, the bacteriophage exalts its virulence toward the invader, and this so much the more rapidly when this bacterium is lacking in resistance and when the bacteriophage is hereditarily the more adapted to the struggle. The more frequent the reinfections by a given bacterium, the more likely is the bacteriophage to quickly acquire a degree of virulence sufficient to inhibit all growth. If the bacterium which invades an individual is the agent of an intestinal infection or if the avenue of infection is intestinal, the infectious process is then prevented at its very inception and the disease aborts before morbid symptoms appear.

The rapid adaptation of the bacteriophage may be delayed or even prevented by unfavorable circumstances. More sensitive than the bacteria to the action of acids and alkalies, the reaction of the medium is a principal factor which influences the development of the bacteriophage and its power of attack. On the other hand, its activity may be annihilated if the invading germ is derived from an organism in which it has been in conflict with the bacteriophage, a conflict which has allowed it to acquire some degree of resistance. In either the one or the other of these cases

the pathogenic bacterium grows and disease results. If the environmental conditions remain unfavorable and inhibit the action of the bacteriophage, the bacterium develops freely and the invaded individual succumbs quickly, or the conflict may become established, with the virulence of the bacteriophage gradually increasing by selection and the resistance of the bacterium likewise increasing as the result of a similar selective process. The condition of the individual in which this conflict is taking place faithfully reflects the changes in the struggle. Convalescence is established only at the moment when the virulence of the bacteriophage effectively dominates the resistance of the bacterium. If the opposite results, if the bacterium acquires a refractory state, no further barrier is opposed to the invasion of the individual and death follows.

In a word, recovery is always a result of the exaltation of the virulence of the bacteriophage, an increase sufficient to permit it to parasitize and destroy the pathogenic bacteria implanted in the body. Death takes place, either as a result of inertia in the bacteriophage, or because of the acquisition of a refractory state by the bacteria, conditions which, in either case, allow the latter to develop without hindrance.

There is, however, a third possibility. One may isolate from the intestinal contents of "bacillus carriers," typhoid or dysentery, and indeed constantly, a resistant pathogenic bacterium and a bacteriophage virulent for this bacterium. There has been a commensality (as is the case with the normal saprophytes of the intestine), a mixed culture. Usually this equilibrium is quickly broken in favor of the bacteriophage and the carrier state ends. But in individuals who become carriers the conflict carried on in the intestine between the bacteriophage and the bacterium is sufficiently long to permit the development of an organic immunity. The bacteria act on the organism only by means of their toxins. A bacterium whose toxin does not exercise any action on the cells of an animal is as inoffensive for it as a bacterium naturally atoxic. From the time when an organic immunity is acquired by the carrier the pathogenic bacterium becomes for him a saprophyte.

One can comprehend, on the other hand, the danger of contamination from carriers, who, although they distribute a viru-

lent bacteriophage also at the same time distribute a resistant bacterium, that is to say, a bacterium particularly apt to negative the defense exercised by the intestinal bacteriophage of the susceptible individuals contaminated by the carrier.

The observations made in pyelonephritis lead us to believe that the individual affected with a chronic infectious disease is in reality an internal carrier. Here also, the individual enjoys an antitoxic immunity. Nevertheless there is a struggle within the organism between the virulent bacteriophage and the resistant bacterium. For while in the intestine the bacterium is henceforth inoffensive and offers no great inconvenience to the host, the presence of a bacterial culture within the tissues is not an indifferent matter because of the inflammatory reactions which it provokes. In addition, phagocytosis is not able to play an active rôle, for we have seen that bacteria which have acquired a resistance to the bacteriophage are likewise resistant to the phagocytic phenomenon.

The rôle of the bacteriophage is not confined to the intestine. Whatever may be the infectious disease under consideration, there is always the introduction of the pathogenic bacterium into the intestine, either by the digestive path or by the hepatic route. Thus, the intestinal bacteriophage may come in contact with, and acquire a virulence for, the pathogenic bacterium. Furthermore, experiment shows that the bacteriophage may enter the circulation in the case of a septicemia. Hence it may exert its action at any point in the body.

The action of the bacteriophage manifests itself in still another way. Growing at the expense of the bacteria it dissolves them. The bacterial substance, dissolved and modified under the action of the lysins secreted by the bacteriophage, is in a physical and chemical state particularly suited to act upon the cells of the organism which elaborate the antitoxins.

Finally, experiment shows that the bacteriophage exercises a preponderant action on phagocytosis. On one side is the fact that the bacteriophage through its lysins possesses an extremely high opsonic power[1] and on the other side, a bacterium

[1] May not the lysins of the bacteriophage and the antilysins of the bacteria be synonyms for opsonins and aggressins?

resisting the bacteriophage is, by this fact, also resistant to phagocytosis.

The bacteriophage, the direct agent of antimicrobial immunity which is by its nature heterologous, at least in the sensitive animal, is also indirectly an agent of organic immunity, by nature homologous.

The history of the disease is in effect the history of the conflict between the bacteriophage and the bacterium. We can observe that the same facts hold true, but on a larger scale, in the history of an epidemic. The virulent ultramicrobe, which is present in the intestine of all convalescents, is disseminated by them with their dejections. It is then capable of "contaminating" susceptible neighboring individuals quite regardless of whether the disease with which it is associated is intestinal, septicemic, or localized in its nature.

Observation shows that in the last analysis the history of an epidemic registers the variations in the struggle between the two agents, the pathogenic bacterium and the bacteriophagous ultramicrobe. It is also clear that the latter is transmissible from individual to individual. The immunity is contagious in the same degree as is the disease itself. The beginning of an epidemic is marked by the diffusion of a bacterium whose virulence is increased progressively by passages through susceptible individuals. Thus the epidemic extends. In its turn the ultramicrobial bacteriophage increases in virulence for the pathogenic bacterium, and extends equally. The epidemic ceases when all susceptible individuals have been infected by the virulent bacteriophage.

We have seen that the bacteriophage may conserve for a long time a "latent" virulence for a given bacterium. These latent virulences, maintained moreover by accidental contaminations, explain the difference which exists between the mode of propagation of sporadic diseases and of epidemic diseases. Against the bacteria, agents of the first, the bacteriophage is always ready to intervene, and it is only exceptionally that infection is followed by disease. In the second, on the contrary, particularly since the agent is most often imported, the bacteriophage does not at the beginning possess a specific virulence. The epidemic extends

and only stops, or assumes a sporadic character, when there has been a diffusion of a bacteriophage virulent for the pathogenic bacterium.

The bacteriophagous ultramicrobe virulent for a given bacterium is cultivable *in vitro*. It is therefore possible to obtain it in any desired amount. If its protective power is real a susceptible individual should be rendered immune by inoculation, just as though he had naturally resisted the contagion. This has been demonstrated to be the case in the experiments made on avian typhosis and in hemorrhagic septicemia in the buffalo.

The injection of an individual with a culture of the bacteriophage virulent for a given bacterium is harmless and causes no reaction, even when the bacteriophage has developed at the expense of a highly toxic bacterium—*B. dysenteriae* or *B. pestis*, for example. The injected ultramicrobes pass quickly into the intestine.

The injection of a culture of the bacteriophage provokes two types of immunity, heterologous and homologous.

The heterologous antimicrobial immunity is effective immediately. Indeed, it exists simply by virtue of the presence in the body of a bacteriophage active for the causative bacterium. In an uncontaminated or non-epidemic area this immunity is transitory. In a contaminated or epidemic area it persists as long as reinfections occur.

The homologous, or organic immunity, develops after an incubation period. It results from the production of specific antibodies, most likely substances similar to the antitoxins. These antibodies are detectable in the serum of the immunized animal and persist there for a period at present undetermined (more than seven months in the case of avian typhosis). The period of incubation in the homologous immunity is the longer as the dose injected is greater.

The immunization experiments against barbone have shown us the importance of the question of dosage. The quantity of bacteriophage culture necessary and sufficient to provoke organic immunity ought, in all cases, to be injected at a single time. As to the dose itself, it must certainly vary in accordance with the disease under consideration, and, consequently, with the

bacterium for which the bacteriophage injected is virulent.[2] In hemorrhagic septicemia of the buffalo the optimum single dose is a quarter of a cubic centimeter per hundred kilograms of body weight. This question of dosage must be fixed by preliminary experiments for the other diseases.[3]

With disease once declared, the introduction into the patient of the ultramicrobe virulent for the causative bacterium ought to place the affected individual in a condition analogous to that of the convalescent individual. The experiments in avian typhosis and in human dysentery show in effect that the ingestion or the injection of cultures of the bacteriophage exert a curative action.

The administration to a patient of an active culture of bacteriophage ought, as may be conceived, to be made at a time as near as possible to the beginning of the disease. For this there are two reasons.

1. We have seen that the acquisition of virulence in the bacteriophage only represents one side of the question of recovery. The bacterium may acquire a state of resistance such that the action of the bacteriophage may be rendered inoperative. The administration of a culture of the active bacteriophage should have the more effect when the resistance of the bacterium is the least. On the other hand, the acquisition of resistance is the result of the conflict within the individual. Thus, the more rapid the intervention the less likely will be the formation of a resistant bacterial race.

2. If there exists at the time of intervention organic lesions incompatible with life the issue of the disease can not be other than fatal whatever the power of the bacteriophage.[4]

[2] It should be noted that here we are dealing with *injection* only. The ingestion of cultures of the bacteriophage does not appear to be attended by the development of an organic immunity. Ingestions can be repeated without inconvenience, as I have demonstrated on myself.

[3] It should be emphasized that the cultures of the bacteriophage used in immunization should be perfectly limpid; that is to say, the lysis ought to be complete. Filtration through a bougie is *essential*, for the reason which we have seen. If necessary, filtration may be replaced by heating at 58°C., but filtration is to be preferred.

[4] The cases recently described by Beckerich and Hauduroy, which were mentioned in the note at the end of the preceding chapter, corroborate

To summarize: Observation and experiment agree in showing that the bacteriophage is the direct agent of antibacterial immunity in the sensitive animal. It dissolves the bacteria at the expense of which it reproduces itself, and does this by means of lysins which it secretes and which remain in the solution once the bacteria are destroyed. These lysins enjoy, furthermore, an extremely high opsonic power, which may likewise contribute, in certain cases, to the destruction of the pathogenic bacteria.

The bacteriophage also contributes to the establishment of organic immunity. The bacterial substance dissolved under the action of the lysins is in a physical and chemical state such that an extremely minute quantity suffices to provoke the formation of a potent organic immunity.

this statement. I state precisely then, and I insist on this point, that the treatment by the bacteriophage of any case of acute infection ought to be undertaken at once, *without the loss of a minute.* Whatever may be the disease it is useless and dangerous to await the results of laboratory examinations destined to confirm the clinical diagnosis. This last ought to be considered as sufficient to warrant the administration of the bacteriophage. Such practice does not incur *any risk* whatever, even though there has been an error in the diagnosis, for the injection or the ingestion of cultures of the bacteriophage is in all cases absolutely innocuous. I would say further, even if the clinical diagnosis proves erroneous the administration of a bacteriophage avirulent for the causative bacterium may be useful. While in Indo-China, at three different times, I administered to cholera patients, *per os,* two cubic centimeters of an anti-Shiga bacteriophage, and two of the three cases recovered. I do not affirm that this fortunate result could be referred to the administration of the anti-Shiga bacteriophage, although there is a strong presumption in favor of this hypothesis. In fact, of the 113 cases of cholera which I observed during my stay there, I did not see a single case recover spontaneously. In any event, even if it be but a coincidence, it is possible to affirm that the administration of the bacteriophage caused no harm in the cholera cases.

In so far as typhoid fever is concerned, for example, I would recommend the removal of the blood necessary for culture at the entrance of the patient into the hospital (or at the first visit of the physician treating the case) and the immediate administration of a culture of bacteriophage. Either two or five cubic centimeters may be given *per os,* or one cubic centimeter may be injected subcutaneously. In this way a bacteriologic diagnosis may be established without necessitating delay in treatment. In a word, whatever may be the disease, the absolute principle ought to be *"never lose a minute."*

The immunity acquired as the result of a single injection of a small quantity of bacteriophage culture is accompanied by the appearance in the blood of a protective principle. The animal which receives this blood enjoys a solid immunity, specific in nature, and identical with that possessed by the animal which received the immunizing injection of bacteriophage. The protective principle is probably an antitoxin. It is possible that this new method of obtaining immunizing sera offers a means of intervening in the course of a disease, even in cases where the administration of the active culture of bacteriophage may be without effect because of the previous acquisition of a resistance by the bacterium.

Our knowledge of the bacteriophage, a cultivable agent of immunity, allows us to entertain the possibility of collective intervention in epidemics.

Whatever may be the epidemic (provided, of course, the agent is known and cultivable) we have first the possibility of individual vaccination by means of a single injection of a small quantity of bacteriophage culture active for the causative bacterium. But we have seen that the presence in the intestine of active ultramicrobes assures the protection of a susceptible individual. We are then able to consider the possibility of collective immunization of the population, for it would be easy to mix cultures of the bacteriophage with the drinking water, especially in urban centers. One might then be assured of an active bacteriophage in the intestine of all susceptible individuals throughout the critical period. The method offers no risk; the cultures can be ingested without inconvenience in any quantity.

In spite of the fact that I have specified at several times in the course of this work that the experiments undertaken deal only with antibacterial immunity in *the susceptible individual*, I want in closing, in order to avoid confusion, to say a few words on the subject of phagocytosis, for it would be strange if, speaking of antibacterial immunity, I made no allusion to this mode of defense.

I do not oppose any of the conclusions of Metchnikoff touching the rôle of phagocytosis in the natural immunity which characterizes the refractory state. In acquired immunity also, Metchni-

koff and his collaborators affirm that phagocytosis plays a capital rôle. That the elimination of bacteria is effected by phagocytosis, *once organic immunity is established,* would appear to be the proper interpretation.

However, in the one or in the other case, the bacteriophage manifests its action. Its activity is not naturally limited to the bacteria pathogenic for a given animal; it is exercised without distinction against bacteria pathogenic and saprophytic, in all circumstances, and in all animals. Even if, in the immune animal, the bacteriophage should remain inert, the bacteria would none the less be eliminated by phagocytosis.

What then is the rôle of the bacteriophage in immunity? The defense of the *susceptible* individual exposed to infection, and the protection of the organism in the course of natural disease. Parasitic of bacteria, the bacteriophage intervenes *directly* to destroy the pathogenic bacteria which venture to invade the organism; secreting lysins endowed with a powerful opsonic action, it renders possible the education of the phagocyte and introduces the establishment of *organic antibacterial immunity;* dissolving the bacteria, it transforms the bacterial substance and places it in a physical and chemical state where it can stimulate the cells of the body which produce the antitoxic antibodies, it introduces thus, the establishment of *organic antitoxic immunity.*

In other terms, the bacteriophage plays a preponderating rôle in all the phenomena of immunity which are accomplished in a susceptible individual. As a result of its presence it follows that, although exposed to infection it is possible to remain unharmed; and although sick, it is possible to recover.

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